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Tissue and serum loss of metalloproteinase inhibitors in high grade soft tissue sarcomas

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Summary. The activity of matrix metalloproteinases (MMPs) in degrading extracellular matrix is controlled by activation of pro-enzymes and inhibition of MMP tissue inhibitors (TIMPs).

To assess proteolytic cascade imbalance in malignancy progression, the enzymatic activity of MMP2 and MMP9 and the expression and serum level of their inhibitors, TIMP2 and TIMP1 respectively, was evaluated in selected patients with high-risk soft tissue sarcoma (STS). Gelatinase activity and inhibitor expression was evaluated on 69 biopsies by zymography and immunohistochemistry. TIMP1 and TIMP2 serum concentration was tested in 53 STS patients and in 56 controls using a sandwich enzyme immunoassay.

Clinical and biological variables were related to clinical outcome of the patients.

A significant gelatinolytic activity was seen in a high percentage of STS. TIMP expression was weak or negative in the majority of samples. The difference between disease-free (p=0.001) and overall survival (p=0.007) curves based on TIMP2 immunoreactivity was statistically significant. TIMP plasma concentration of 53 STS revealed significantly lower levels compared to those of 56 controls (p=0.0001).

In conclusion, low levels of negative regulators of proteolysis may be related to tumor biological aggressiveness and used to select patients with poor prognosis to improve cure.

Key words: Prognosis, Metalloproteinases, Inhibitors, Soft tissue sarcoma

Introduction

Tumor metastasis consists of a series of multiple tumor-host interactions that lead to the invasion of local host tissue by tumor cells that later enter the circulation, arrest at the distant vascular bed, extravasate and then proliferate at the distant organ site (Aznavoorian et al., 1993).

Previous studies have shown that the invasive capacity of tumor cells is related to the presence of MMPs that degrade extracellular matrix components (Carmeliet at al., 1997). MMPs are a family of zinc-dependent endopeptidases. Two members of this family, MMP2 and MMP9, show an elevated expression in many malignant human tumors, and the association with tumor metastasis derives from their ability to degrade basement membrane collagen IV (Ray and Stetler-Stevenson, 1994).

The activation of latent pro-enzymes pro-MMP2 (gelatinase A, 72KDa) and pro-MMP9 (gelatinase B, 92KDa) is regulated by the TIMP2 and TIMP1 inhibitors respectively. TIMP1 is a glycoprotein with a molecular mass of about 28 KDa which can form a complex with 92 KDa type IV collagenase. TIMP2 is a 21 KDa protein capable of binding to both the latent and activated forms of gelatinase A. An unbalance in this proteolytic system may contribute to tumor invasiveness and metastasis (Gomez et al., 1997).

Soft tissue sarcomas are a heterogeneous group of tumors with a common mesenchymal origin. They represent <1% of cancers and from 7% to 10% of childhood cancers. Fifteen to twenty percent of these tumors are poorly differentiated and pose diagnostic problems (Campanacci, 1999). Apart from diagnostic criteria, the identification of new prognostic markers allows the differentiation of histologically and clinically atypical subtypes and the selection of high-risk patients to whom administer adequate therapies.

A high percentage of malignant STS develop long-term distant metastasis. Recently, an Italian randomized trial demonstrated a positive impact of intensified adjuvant chemotherapy on disease-free survival (DFS) and overall survival (OS) of patients with high-risk extremity STS (Frustaci et al., 2001). The present study evaluated the expression of pro-MMP2 and pro-MMP9 by gelatin zymography, as well as the immunoreactivity and serum level of their inhibitors TIMP2 and TIMP1, in a patient population with high-risk STS, in order to

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assess the significance of proteolytic cascade unbalance in malignancy progression.

Materials and methods

The study was carried out on 69 biopsies from patients with high grade primary STS.

Tumor samples included 15 pleomorphic liposarcomas, 19 synoval sarcomas (SS), 7 malignant peripheral nerve sheath tumors (MPNST) and 28 malignant fibrous histiocytomas (MFH). For each sample both frozen and paraffin-embedded material was available. All tumors were diagnosed at the Rizzoli Institute by a group of expert pathologists. Diagnoses were based on hematoxylin-eosin-stained samples. For differential diagnosis, immunohistochemical analysis with specific antibodies was performed. Selection criteria were primary tumors deeply localized with a diameter greater than 5 cm, high grade III and IV according to Broders' criteria (Broders et al., 1939), spindle cell or polymorphous sarcomas, no local relapse and no previous radio/chemotherapy.

To test plasma TIMP1 and TIMP2 concentration, blood samples were collected from 53 patients before treatment. Histological type included 15 liposarcoma, 13 SS and 25 MFH. 56 healthy subjets were included as control group.

Immunohistochemistry (IHC)

Immunodetection of TIMP1 and TIMP2 was performed respectively with 102D1 MAb (Ab-2, Neomarkers, Fremont,CA, dilution 1:100) and 2TMPO4 MAb (Ab-4, Neomarkers, dilution 1:50).

Paraffin sections were prepared from formalin-fixed paraffin-embedded tumor samples. Sections were cut at 5 μm, deparaffinized by xylene and rehydrated in graded alcohols and water. Microwave treatment using 10 mM citric acid solution (pH 6) was performed (3 cycles/5 min at 750W) before treatment to eliminate endogeneous peroxidase and blocking serum. Sections were incubated at 4 °C overnight with primary antibodies.

After treatment with secondary biotinylated antibody and streptavidin-biotin complex (sABC) (Biomeda, Foster City, CA), the staining was developed in 3-amino 9-ethylcarbazole. The nuclei were counterstained with hematoxylin. Immunoreaction was classified as follows: less than 10% of stained tumor cells (negative); 11-25% (weak); 26-49% (moderate); and more than or equal to 50% (strong). Negative controls were included for each stained series.

Zymography

According to standard procedure (Sambrook et al., 1989), protein extracts were prepared by mincing and homogenizing fresh samples in extraction buffer (50 mM Tris-HCL pH 8, 150 mM NaCl, 1 mM DTT, 50 mM NaF, 0.5% sodio deoxycholate, 0.1% SDS, 1% NP-40, 0.1 mM PMSF, 2μg/ml aprotinin, 1μg/ml leupeptin, 100 mM Na_3VO_4).

Gelatin zymography was performed to analyze the levels of secreted pro-MMP2 and pro-MMP9, using 10% SDS polyacrylamide gels containing 0.1% gelatin. After electrophoresis, gel was washed in 2.5% Triton X-100 and incubated (18 hours, 37 °C) in 0.15 M NaCl, 10 mM $CaCl_2$, 50 mM Tris/HCl.

The gels were stained with 0.1% Coomassie Blue in a solution of 50% methanol and 10% acetic acid. Bands with gelatinase activity, detected as clear bands against the blue-stained gelatin background, were visualized after the gels were destained with 50% methanol and 10% acetic acid, and quantified by using GS-670 imaging densitometer (Bio-Rad, Hercules, CA).

A 1.2 optical density cut-off was chosen to select tumors with significant gelatinase amount.

Enzyme-linked immunoabsorbent assay (ELISA)

Serum TIMP1 and TIMP2 concentrations were measured with a two site ELISA 'sandwich' format (Fuji Chemical Industries, Toyama, Japan) using the anti-human TIMP1 or TIMP2 monoclonal antibodies. Serum samples were diluted 1:50 for TIMP1 and 1:10 for TIMP2 analysis. 100μl of diluted unknown sample was pipetted into the appropriate microplate wells coated with primary antibodies. Microplate wells were incubated with 100μl of horseradish peroxidase-conjugated secondary antibody at room temperature for 1-2 hours. Then, the plates were washed with appropiate buffer and incubated for 30 minutes with 100μl of room temperature-equilibrated 3,3',5,5'-tetramethylbenzidine (TMB)/hydrogen peroxide, in 20% dimethylformamide. The reaction was stopped by addition of 100μl of 1M sulfuric acid, and absorbance was measured at 450 nm. Both plasma concentrations of TIMP1 and TIMP2 were calculated using standard curves.

Patients and statistical analysis

Selected patients with high-risk STS (29 females, 40 males), adequate histological material and complete clinical data were included. The age ranged from 6-80 years, with a median of 50 years.

Clinical follow-up was evaluated considering time to metastasis, disease-free survival (DFS) interval and overall survival (OS). DFS and OS were calculated from the date of diagnosis to the last day of follow-up or event. 23 patients were disease-free, while 46 developed metastasis and of these, 37 died of disease. Follow-up ranged from 3 to 192 months, with a mean of 39 months. Minimal follow-up for disease-free patients was 3 years. Clinical features are shown in Table 1. Twenty-one patients had only radiation therapy, nine only chemotherapy as adjuvant, four had both treatments and thirty-five patients were given no treatment.

Univariate analysis was performed using the chi-square test and analysis of variance. DFS and OS were

assessed by Kaplan-Meier analysis (Kaplan and Meier, 1958) and their prognostic significance by logrank test.

Results

By zymography analysis, 36 (52%) and 34 (49%) out of the 69 tumors had significant levels of pro-MMP2 and pro-MMP9 respectively (Fig. 1). The analysis of variance showed significantly higher mean values of gelatinases in liposarcoma versus MFH (p=0.005 for MMP2, p=0.001 for MMP9), in liposarcoma versus MPNST (p=0.03 for MMP9), in SS versus MPNST (p=0.005 for MMP2) and in SS versus MFH (p=0.0001 for MMP2 and p=0.008 for MMP9).

With regard to MMP tissue inhibitors, the IHC analysis showed that 23/69 (33%) immunostained for TIMP2, with a moderate to strong expression pattern, and 5/69 (7%) were positive to TIMP1 antibody (Fig. 2). No association with prognosis was observed between latent pro-MMPs, TIMP1 expression, therapy, sex, age or site and DFS or OS.

TIMP2 expression was inversely correlated to poor prognosis in terms of both DFS and OS (chi-square =8.35; p=0.003 and chi-square =7.46; p=0.006 respectively). Metastasis rate was 43% (10/23) for patients with TIMP2-positive tumors versus 78% (36/46) in TIMP2-negative tumors.

Concerning mortality, the rate was 30% (7/23) and 65% (30/46) for TIMP2-positive and TIMP2-negative cases respectively.

The difference between DFS curves and OS curves based on TIMP2 immunoreactivity was statistically significant (logrank=10.6; p=0.001, logrank=7.13; p=0.007 respectively) (Figs. 3,4). In the metastatic group, 36/46 (78%) patients lacked TIMP2 expression versus 10/23 (43%) in the disease-free group. Furthermore, 30 out of 37 (81%) patients who died of disease had minimal or no TIMP2 expression.

Plasma concentration of TIMP1 and TIMP2 in 53 STS patients (861.4±47.8 ng/ml and 35.6±9.7 ng/ml respectively) revealed significantly lower mean levels compared to those of 56 healthy controls (1359.4±76.9 ng/ml; p=0.0001 for TIMP1 and 131.4±9.5 ng/ml; p=0.0001 for TIMP2).

45/53 (85%) and 50/53 (94%) STS patients had TIMP1 and TIMP2 concentration levels under the lower limits of normal range respectively (healthy group mean levels ± 2 SD).

By analysis of variance, significantly lower levels were found in liposarcoma (698.3±31.5ng/ml) and SS (663.4 ±36.8 ng/ml) compared to MFH (1062.2±81.4 ng/ml; p=0.01) for plasma TIMP1 and in MFH (7.56±1.5 ng/ml) versus liposarcoma (82±31.3 ng/ml; p=0.001) for plasma TIMP2 (Fig.5,6).

Discussion

Soft tissue sarcomas consist of numerous histotypes with prognosis determined by clinical parameters such as size, location, grading and resection marginal status (Campanacci, 1999). Besides this, the identification of molecular markers is necessary to understand the biological aggressiveness inducing tumor cell spread. Proliferation index Ki67, DNA content, matrix degrading enzymes and deregulation of cell-cycle control are considered prognostically important (Hall et al., 1988; Dutta et al., 1995).

Our previous study on a series of STS with different clinical and histopathological aspects, established an association between MMP2 expression, evaluated by IHC, histological grade and metastatic event (Benassi et

Table 1. Characteristics of 69 patients with STS.

VARIABLES	NUMBER OF PATIENTS	NUMBER OF METASTASES
Sex		
female	29	18
male	40	28
Age (years)		
≤18	5	2
> 18	64	44
Tumor type		
Liposarcoma	15	10
MPNST	7	4
Synovial sarcoma	19	12
MFH	28	20
Therapy		
Only chemotherapy	9	8
Only radio	21	13
chemo + radio	4	2
none	35	23
Anatonic site		
thorax + abdomen	13	9
leg	46	29
arm	4	4
foot	6	4

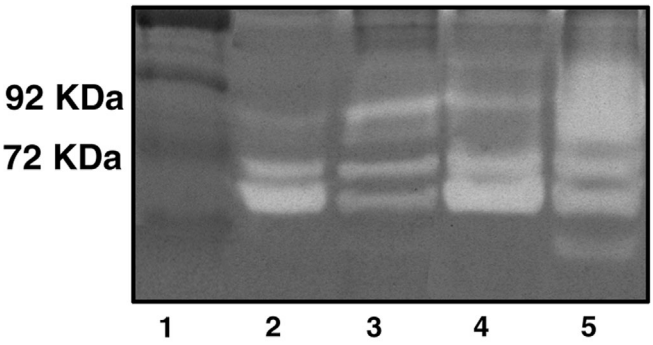

Fig. 1. Zymography of gelatinase expression in tumor samples from patients with STS. The proteins from samples of STS were extracted as descibed in Materials and methods. Electrophoresis (10 μg/lane) was performed on 1% SDS polyacrylamide gels containing 0.1% gelatin. 72KDa and 92KDa bands correspond to pro-MMP2 and pro-MMP9 respectively. Lane 1: high range prestained standard, lane 2: MFH, lane 3: MPNST; lane 4: SS; lane 5; liposarcoma.

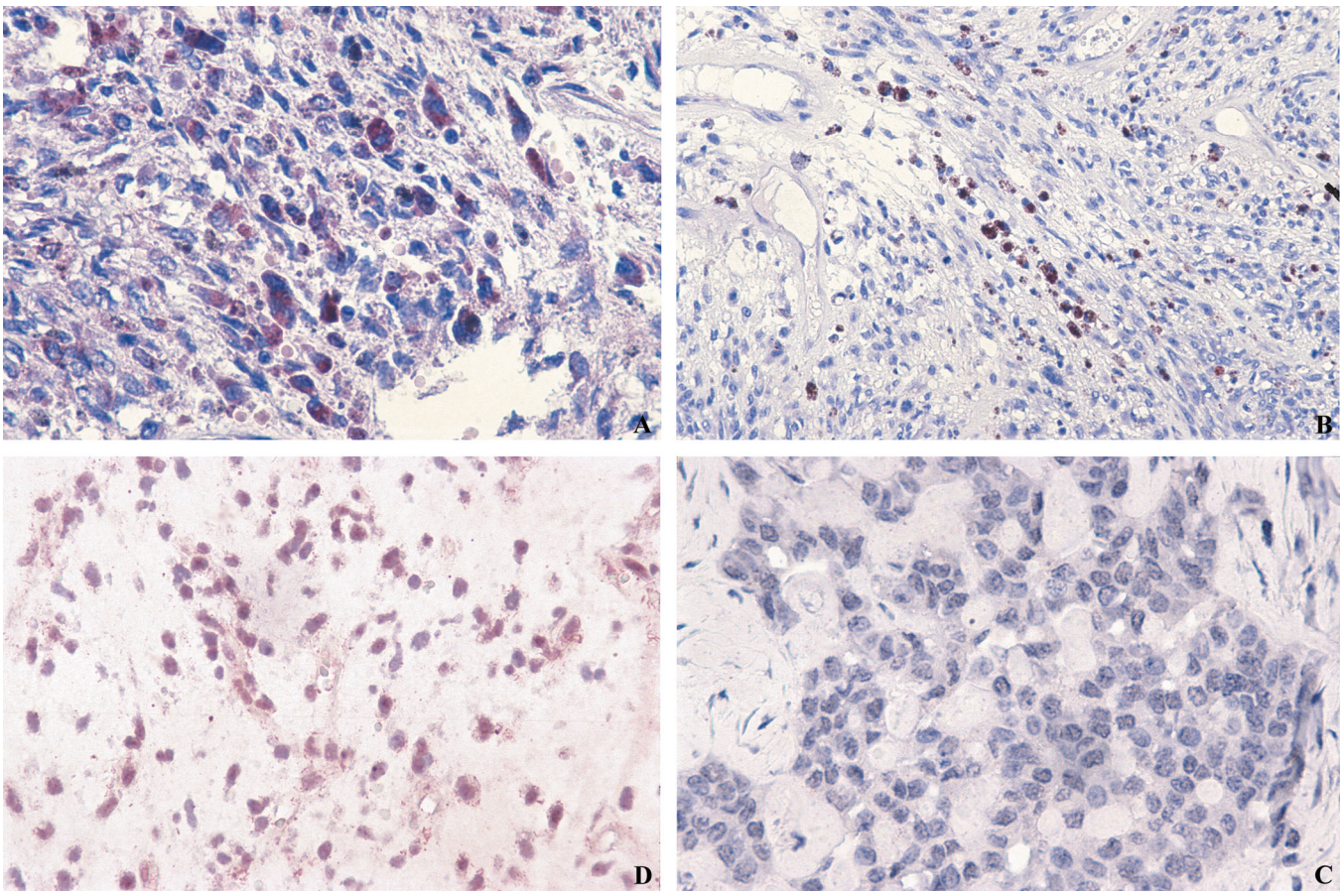

Fig. 2. a. Moderate immunoreactivity of TIMP2 in MFH (IHC, x 40). **b.** Scattered immunoreactivity of TIMP1 in MFH (IHC, x 20). **C.** TIMP2 expression in liposarcoma (IHC, x 40). **d.** Negative expression of TIMP1 in synovial sarcoma (IHC, x 40).

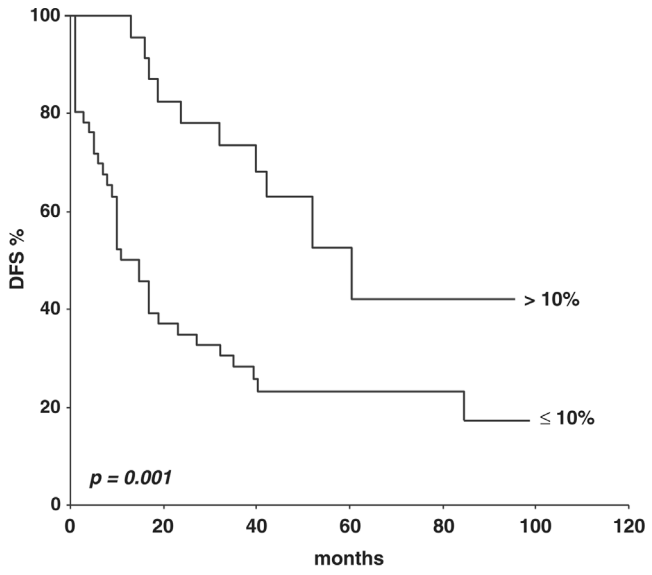

Fig. 3. Disease-free survival curves by TIMP2 reactivity in 69 patients with STS. The patients were divided into two groups using a cut-off of 10%.

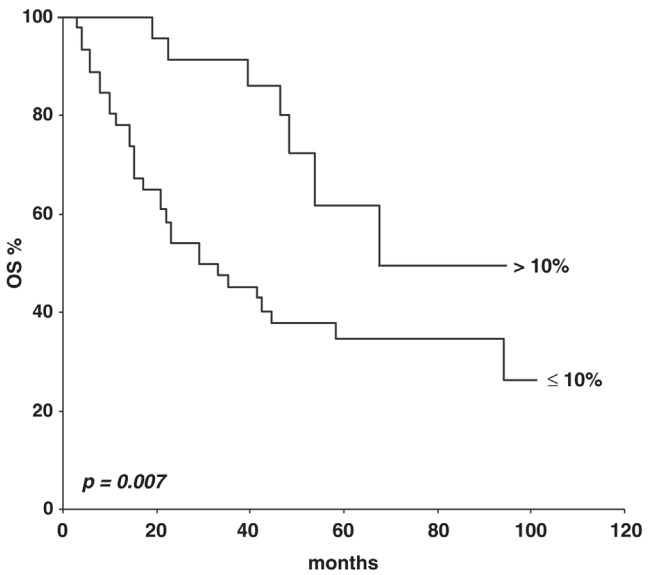

Fig. 4. Overall survival curves by TIMP2 reactivity in 69 patients with STS. To define the relationship between TIMP2 expression and overall survival a cut-off of 10% was used.

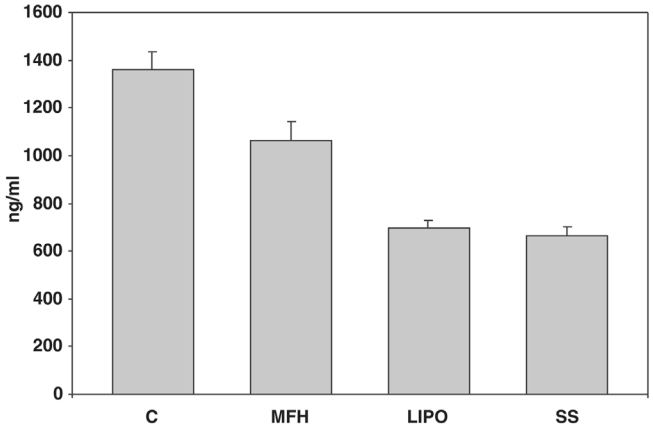

Fig. 5. Mean plasma levels of TIMP1 in control group (C) were significantly higher compared to those in STS patients. Lower levels were found in liposarcoma and SS compared to MFH.

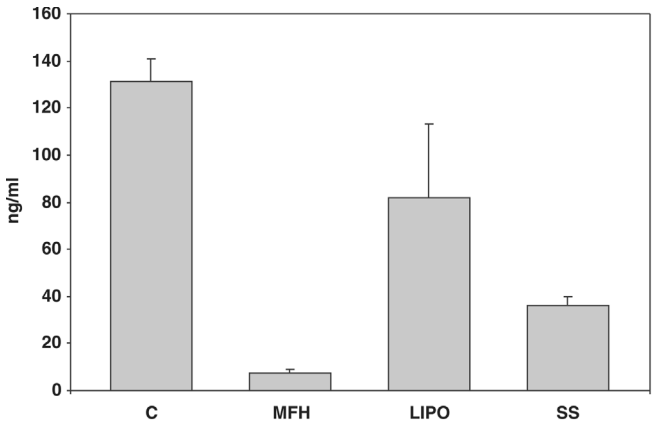

Fig. 6. Mean plasma levels of TIMP2 in control group (C) were significantly higher compared to those in STS patients. The lowest levels were detected in MFH group.

al., 2001).

Other studies have shown that the expression of this enzyme was correlated with the progression of colonrectal, gastric and breast carcinoma (Murray et al., 1996; Sier et al., 1996; Talvensaari-Mattila et al., 1998). However, the invasiveness of malignant cells also depends on low TIMP levels (Coussens and Werb, 1996). The present study showed that pro-MMP2 and pro-MMP9 were strongly expressed in high-risk STS patients. The latent pro-enzyme levels though, were not statistically associated with prognosis or other clinical features, with exception of the histological type. On the contrary, TIMP2 expression was positively and significantly correlated to a more favorable prognosis in terms of DFS and OS. Although a dual function of TIMPs in the regulation of tumor invasion has been demonstrated (Gomez et al., 1997; Airola et al., 1999), these data confirm the predominant tumor invasion inhibitory activity of TIMP2 (Albini et al., 1991; DeClerck et al., 1992), and its important prognostic role in STS (Benassi et al., 2001). Recent findings in hepatocellular carcinoma showed that patients with lower plasma levels of TIMP2 had shorter long-term survival (Giannelli et al., 2002).

The important role of MMP inhibitors on the biological aggressiveness of high-risk STS is supported by the evidence that almost all tumor samples studied lacked TIMP1 expression and by the significant decrease of both TIMP1 and TIMP2 in STS patient plasma compared to healthy controls.

Although this reduction seems to be related to histological subtype, the majority of cases showed TIMP1 and TIMP2 serum levels under the lower limits of the normal range.

In conclusion, we demonstrated low or absent TIMP levels in high-risk STS patients. In detail, the TIMP2 immunoreactivity was inversely correlated to metastatic spread and poorer prognosis. In addition to well-recognized clinical prognostic factors, the investigation of new biological markers involved in tumor invasiveness is necessary to better define high-risk patients and further improve therapeutic strategies. Thus, the high aggresiveness can be explained, at molecular level, by the alteration of factors that negatively regulate the proteolytic cascade.

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