High Serum TIMP-1 is Associated with Adverse Prognosis in Endometrial Carcinoma

MARIA HONKAVUORI[1], ANNE TALVENSAARI-MATTILA[1], ULLA PUISTOLA[1], TAINA TURPEENNIEMI-HUJANEN[2] and MARKKU SANTALA[1]

Departments of [1]Obstetrics and Gynecology, and [2]Oncology and Radiotherapy, University of Oulu, Oulu, Finland

Abstract. *Background: Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) play a key role in extracellular matrix (ECM) turnover and remodeling. Changes in their expression levels have been observed in various tumor types. However, their clinical significance and prognostic importance in the progression of endometrial carcinoma is still unclear. This study aimed to evaluate the circulating levels of gelatinases and tissue inhibitors of gelatinases, and to study their relationships with the clinical behavior of endometrial cancer. Materials and Methods: Pretreatment serum levels of MMP-2, MMP-9, TIMP-1, TIMP-2 and MMP-2/TIMP-2 complex were quantitatively measured by enzyme-linked immunosorbent assay (ELISA) in 93 patients presenting with primary endometrioid endometrial adenocarcinoma. The study population was divided into low-risk and high-risk patient groups as determined by conventional prognostic criteria. Results: Elevated serum levels of TIMP-1 at diagnosis were found in the high-risk patient group (p=0.018). The median follow-up time was 101 months. A cut-off value of 536 ng/ml was used to divide the serum values of TIMP-1 into two groups. A high serum concentration of TIMP-1 was associated with shortened relapse-free (p=0.036) and cancer-specific survival (p=0.029). Conclusion: The results suggest that the preoperative serum TIMP-1 level predicts the behavior of endometrial cancer. However, in multivariate analysis TIMP-1 was not an independent prognostic factor.*

Proteolytic degradation of the extracellular matrix (ECM) is a fundamental aspect of cancer development and a key event in the regulation of tumor proliferation and metastasis. Matrix metalloproteinases (MMPs) constitute a family of zinc-dependent endopeptidases that are capable of degrading most components of the basement membrane and ECM, facilitating cell migration (1). On the basis of their substrate specificities and sequence characteristics, they can be divided into four main classes: collagenases, gelatinases, stromelysins and membrane-type MMPs (2, 3). Matrix metalloproteinases, especially gelatinases MMP-2 and MMP-9, have been shown to be involved in connective tissue remodelling in normal processes, such as tissue repair, embryonic development and menstruation, but have also been implicated in a variety of pathological conditions, including atherosclerosis, arthritis as well as tumor invasion and metastasis (3-7).

Tissue inhibitors of matrix metalloproteinases (TIMPs) are considered some of the most important regulators of metalloproteinase activity. Four TIMPs have been characterized in humans and designated as TIMP-1, -2, -3 and -4. TIMPs are natural inhibitors of MMPs and form noncovalent complexes with them that are resistant to heat denaturation and proteolytic degradation (8, 9). TIMP-1 binds MMP-9 in particular, whereas TIMP-2 mainly binds to MMP-2 (10, 11). In addition to their inhibitory role, TIMPs can also take part in the activation of MMPs. They further seem to have antiangiogenic activity and to be able to act as growth factors (12). The role of TIMPs in cancer progression is still uncertain. It seems that TIMPs inhibit tumor growth and invasion in some tumors, whereas in certain types of malignant tumors high TIMP levels correlate with more aggressive behavior.

To date, no studies have been published in the English language literature concerning the role of circulating matrix metalloproteinases in endometrial cancer. The present study aimed primarily to evaluate the circulating levels of gelatinases and tissue inhibitors of gelatinases and secondarily to study their relationships with the clinical behavior of endometrial cancer.

Materials and Methods

Patient material. The study group consisted of 93 patients diagnosed with endometrial cancer and treated in the Department of Obstetrics and Gynecology, Oulu University Hospital, between 1992 and 1997.

Correspondence to: Markku Santala, MD, Ph.D., Department of Obstetrics and Gynecology, PO Box 5000, 90014, University of Oulu, Oulu, Finland. Tel: +358 8 3153586, Fax: +358 8 3154310, e-mail: markku.santala@oulu.fi

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According to the FIGO criteria, there were 69 stage I, 8 stage II, 14 stage III and 2 stage IV cancers. Forty-six tumors were well (grade 1), 34 moderately (grade 2) and 13 poorly (grade 3) differentiated. Only endometrioid adenocarcinomas were included. In most cases, the primary treatments were extrafascial hysterectomy, bilateral salpingo-oophorectomy and pelvic lymphadenectomy. One patient had preoperative chemotherapy and 27 patients postoperative adjuvant cisplatin-based chemotherapy. Thirteen patients had postoperative vaginal cuff brachytherapy and 38 patients postoperative external whole pelvic irradiation. Six patients had both internal and external radiation therapy.

The study population was divided into low-risk and high-risk patient groups based on conventional prognostic parameters. The low-risk group included patients with stage Ia-Ib and histological grade 1-2 disease (n=47). The high-risk group included patients with disease of higher stage and histological grade 3 or showing either vascular or lymphatic invasion (n=46). The median age of the low-risk patients was 47 years (range 39-84), whereas the corresponding age of the high-risk patients was 66 (range 37-86) (p=NS). The median body mass index (BMI) was 27 kg/m^2 for the low-risk patients (range 20-54) and 28 kg/m^2 (range 20-41) for high-risk patients (p=NS). The median follow-up time was 101 months (range 0-144 months). Venous blood samples were collected prior to surgery. Sera were obtained by centrifugation without using any artificial coagulation activator and stored frozen at –20˚C until analysis for this study.

All research was conducted with the patients' informed consent to have their venous blood samples banked for future studies for biological cancer markers and for use of their clinical data. The study was approved in the Ethical Committee of the Oulu University Hospital.

ELISA assays for MMP-2, MMP-9, TIMP-1, TIMP-2 and MMP-2/TIMP-2 complex. The concentrations of MMP-2, MMP-9, TIMP-1, TIMP-2 and MMP-2/TIMP-2 complex in the serum of the patients with endometrioid adenocarcinoma were determined by enzyme-linked immunosorbent assay (ELISA). ELISA assays were performed on 8-well EIA/RIA microtitre plates (Corning Inc., Corning, NY, USA) using standard protocols (13). Standard samples were included in every plate and the standard curves were required to be similar in each lot. All measurements were performed in duplicate.

The wells were coated overnight at 4˚C with a specific monoclonal antibody provided by SBA Sciences, Oulu, Finland (code DB-102 for TIMP-1, code T2-101 for TIMP-2 and MMP-2/TIMP-2, code Ge-213 for MMP-9). Following coating, diluted serum samples and standards for TIMP-1, TIMP-2 and MMP-2/TIMP-2 were incubated for 60 minutes, or overnight in the case of MMP-9. Non-specific binding was blocked with phosphate-buffered saline containing 1% bovine serum album (BSA-PBS). The wells were thoroughly washed before each stage of the procedure, in the first phase with PBS and at the later stages with PBST (0.05% Tween 20 in PBS). The bound proteins were detected with polyclonal antibodies against each of the analytes (anti-TIMP-1, code DB-205 for TIMP-2, code DB-202 for MMP-2/TIMP-2, code DB-209 for MMP-9) (SBA Sciences, Oulu, Finland). A peroxidase-conjugated anti-chicken antibody (Chemicon International, CA, USA) was used to detect the bound polyclonal antibody, and an OPD solution (*o*-phenylenediamine dihydrochloride, P-1526; Sigma, Steinheim, Germany) was used to visualize the peroxidase conjugate. The reaction was stopped with 1.8 M H_2SO_4. Color formation was measured at 492 nm with a microplate reader (Anthos Reader 2001; Anthos Labtec Instruments, Walls, Austria) using the Windows-based control and evaluation software for Rosys Anthos microplate readers (Anthos Labtec Instruments). The sensitivity of the assays was 2 ng/ml for MMP-9, 1 ng/ml for TIMP-1, 2 ng/ml for TIMP-2 and 2 ng/ml for MMP-2/TIMP-2 complex.

Table I. *Serum levels (ng/ml) of MMP-2, MMP-9, TIMP-1, TIMP-2 and MMP-2/TIMP-2 complex in endometrial carcinoma patients.*

Protein	Number of patients	Low risk	High risk	*P*-value
s-MMP-2	93	1220 (438-2190)	1270 (672-1860)	0.337
s-MMP-9	92	155 (51-376)	141 (62-446)	0.585
s-TIMP-1	90	441 (257-1100)	517 (271-918)	0.018
s-TIMP-2	93	354 (210-601)	362 (211-578)	0.449
s-MMP-2/TIMP-2	93	525 (226-908)	573 (293-848)	0.051

Serum MMP-2 concentration was determined by using human MMP-2 ELISA (code RPN2617; Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. This assay recognizes the precursor of MMP-2 (proMMP-2), *i.e.* free proMMP-2 and that complexed with TIMP-2, but not the active form of MMP-2. The sensitivity of the assay for MMP-2 was 0.37 ng/ml.

Statistical analysis. Statistical analysis was carried out using the SPSS program (Statistical Analysis System, Chicago, IL, USA) for Windows. The associations between the clinicopathological categorical variables and MMP-2, MMP-9, TIMP-1, TIMP-2 or MMP-2/TIMP-2 complex serum concentrations measured by ELISA were assessed with Fisher's exact test. For continuous variables not normally distributed, the Mann-Whitney *U*-test was used. The overall survival and relapse-free survival rates were assessed by the Kaplan-Meier method. The differences in survival between the subgroups were compared by means of a log-rank test. Survival was defined as the time from the primary operation to the date of death or the last control visit. *P*-values less than 0.05 were considered significant.

Results

We found elevated serum levels of TIMP-1 in the high-risk patient group. The median concentration of TIMP-1 was 441 ng/ml in the low-risk patient group compared to 517 ng/ml in the high-risk patient group. The difference was statistically significant (p=0.018). The MMP-2/TIMP-2 complex was also elevated in the high-risk patient group compared to the low-risk group. However, the difference did not quite reach statistical significance (p=0.051). In TIMP-2, the serum concentrations did not differ between the patient groups, nor were there any statistically significant differences between the groups in the levels of MMP-2 or MMP-9 (Table I).

None of the serum levels of MMPs or the levels of TIMPs were associated with the clinical stage of the disease, with the histological grade of the tumor, or with age.

By the end of the study, sixteen patients had died of endometrial carcinoma. None of the patients with low-risk

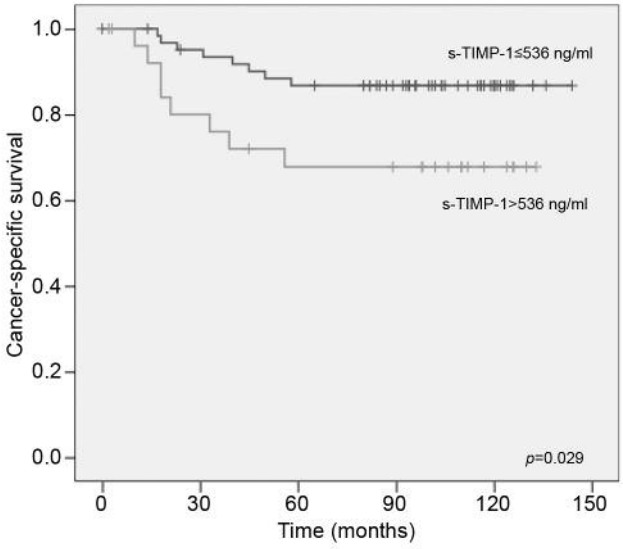

Figure 1. *Cancer-specific survival according to preoperative serum TIMP-1 (s-TIMP-1 ≤536 ng/ml, n=63; s-TIMP-1 >536 ng/ml, n=27).*

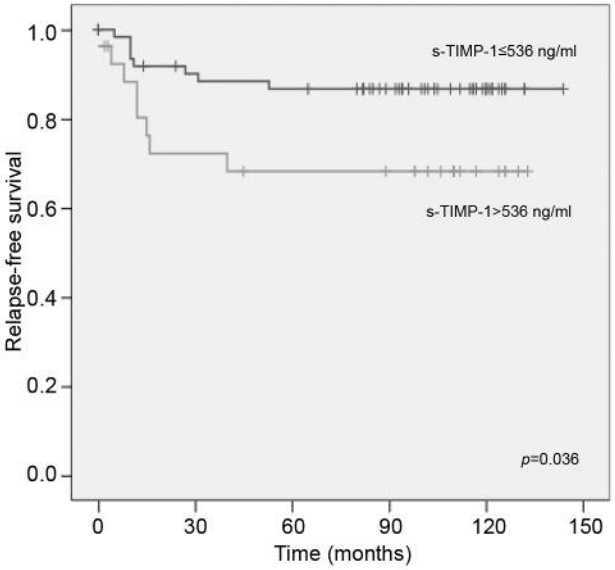

Figure 2. *Relapse-free survival according to preoperative serum TIMP-1 (s-TIMP-1 ≤536 ng/ml, n=63; s-TIMP-1 >536 ng/ml, n=27).*

tumors died of their disease during the follow-up. For survival analyses, we determined the cut-off value for TIMP-1 by utilizing the receiver operating characteristic (ROC) curve. A cut-off value of 536 ng/ml was used to divide the serum values of TIMP-1 into two groups. The preoperative serum TIMP-1 level was found to be associated with cancer-specific survival (p=0.029) (Figure 1). In both groups, 8 patients died of endometrial carcinoma. The Kaplan-Meier analysis showed that the 5-year cancer-specific survival rate of the patients with high TIMP-1 values was 68%. The corresponding figure for the patients with low TIMP-1 values was 87%. A statistically significant correlation was also found between relapse-free survival and serum TIMP-1 levels (p=0.036) (Figure 2). Neither the pretreatment serum MMP-2, MMP-9, TIMP-2 or MMP-2/TIMP-2 complex concentration was associated with the time of relapse or cancer-specific survival.

Discussion

In this study, we show that a high preoperative serum TIMP-1 concentration may be prognostic of shortened survival in endometrial cancer. This is the first study to show a statistically significant difference in survival associated with circulating TIMP-1 in this disease. Though the range of serum values of TIMP-1 was wide, the correlation between high preoperative serum TIMP-1 concentration and poor survival seems evident.

The role of TIMP-1 in tumor progression is unestablished. TIMP-1 is considered to have a growth- and metastasis-inhibiting capacity (8, 9). However, some recent studies of other cancer types have supported the role of high TIMP-1 as a marker of aggressiveness and poor survival. Elevated levels of circulating TIMP-1 have previously been associated with poor prognosis in ovarian, breast, head and neck, lung and colorectal carcinoma (11-17). As regards endometrial carcinoma, there are no data concerning serum or plasma TIMP-1 and the progression of the cancer. Tissue TIMP-1 has been reported to predict independently shortened survival in multivariate analysis in breast carcinoma, and similar results have also been reported in renal cell carcinoma, head and neck squamous cell carcinoma and ovarian carcinoma (1, 15, 18).

The use of serum has been questioned, especially regarding MMP-9 as it is suspected that MMP-9 is released in excess from blood cells during serum sampling, leading to unrealistically high MMP-9 concentrations (19). In recent work by Kuvaja *et al*. (20), sample type was found to have an effect on the concentrations of metalloproteinases and their inhibitors in circulating blood. However, the serum and plasma TIMP-1 values were highly correlated, justifying the use of serum in TIMP-1 tumor marker studies as long as the generally higher levels in serum are acknowledged. Pro-MMP-9 levels were significantly affected by the presence of blood coagulation activator in the serum sample and MMP-9 was therefore considered more reliably determinable in plasma samples. In our study, however, no coagulation activators were used.

In this study, we did not find a statistically significant correlation for the serum MMP-2/TIMP-2 complex in the low-risk and high-risk patient groups, even though there seemed to be obvious differences in the MMP-2/TIMP-2 values between

the patient groups. The *p*-value was, however, 0.051, and it remains possible that the difference found is clinically relevant, but the number of patients is too small in this study to show a clear statistical correlation. For measuring the MMP-2/TIMP-2 complex, the plate is coated with monoclonal anti-TIMP-2 antibody and the bound complex is detected with polyclonal anti-MMP-2 antibody. Therefore, it only detects complexes that have one MMP-2 and one TIMP-2 molecule thus the possibility for cross-reaction in relation to free TIMP-2 or MMP-2 is excluded. The role of the circulating complex is still questionable since a recent study by Vasala *et al*. (21) showed low MMP-2/TIMP-2 complex levels to be associated with poor prognosis in patients with bladder cancer. Kuvaja *et al*. (22) found no associations with the serum proMMP-2/TIMP-2 complex and relapse-free or cancer-specific survival. Hoikkala *et al*. (23) reported a correlation of higher serum levels of the proMMP-2/TIMP-2 complex with lower stage of the disease and enhanced survival.

We did not find any correlation between the MMP-2, MMP-9, TIMP-1, TIMP-2 or MMP-2/TIMP-2 complex serum levels and the conventional prognostic indicators of endometrial cancer. MMP-2 has especially been associated with unfavourable prognostic factors in many cancer types (24-27). Only one study has so far reported circulating matrix metalloproteinases in endometrial carcinoma. Adamiak *et al*. (28) found serum MMP-2 levels to be statistically higher in clinically advanced stages of endometrial carcinoma in a small (n=30) Polish patient series, of which study only the abstract is available in English. Furthermore, no survival analyses were done.

An advantage of our study is that all patients were operated upon and treated in the same gynecological oncological unit in a university hospital, and all of them were surgically staged according to the FIGO criteria. Only endometrioid endometrial adenocarcinomas were included, which makes the patient series quite homogenous. The patients were also followed up systematically after the operation and the follow-up time was long. Histological specimens were evaluated by experienced doctors specialized in gynecological pathology. The study population was quite small, however, which might have caused the low power of significance in the analysis of MMP-2/TIMP-2 complex levels. Another confounding factor may be due to the age differences of the patients. The patients with poor prognosis were evidently older, although the difference was not statistically significant. On the other hand, age is an independent risk factor in many types of cancer, including endometrial carcinoma.

Taken together, preoperative serum measurement of the TIMP-1 concentration might be beneficial in deciding about the primary adjuvant treatment in endometrial carcinoma or in the follow-up of patients treated for endometrial cancer. Serum MMP-2, MMP-9, TIMP-2 and possibly MMP-2/TIMP-2 complex measurements, on the other hand, do not seem to yield useful clinical data. Further studies on larger study populations are needed to clarify the prognostic role of TIMP-1 as well as the function of the preoperative serum MMP-2/TIMP-2 complex in endometrial cancer.

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