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RESEARCH ARTICLE

Immunoreactivity for TIMP-2 is associated with a favorable prognosis in endometrial carcinoma

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Abstract Tissue inhibitors of metalloproteinases are important regulators of metalloproteinase activity, and the balance of active enzyme and inhibitor is a critical determinant of tumor cell invasiveness. This study aimed to evaluate the prognostic and clinical implications of the two main inhibitors of matrix metalloproteinases, TIMP-1 and TIMP-2, in endometrial carcinoma. The material consisted of 241 patients with primary endometrial carcinoma. The median follow-up time was 77 months. Expressions of TIMP-1 and TIMP-2 proteins were examined in paraffin-embedded tumor sections by immunohistochemical methods. Positive staining for TIMP-1 and -2 was observed in 88% and 86% of the primary tumors, respectively. The Kaplan–Meier analysis showed that the 5-year cancer-specific survival rate of the patients with TIMP-2 positive immunostaining was 89% and that of the TIMP-2 negative patients 78%. Positive immunoreaction for TIMP-2 correlated with favorable cancer-specific and overall survival. When including only endometrioid adenocarcinomas, a similar trend towards favorable survival was seen. Excluding stage IA carcinomas, the difference became again statistically significant. For TIMP-1, there was no statistically significant association with overall or cancer-specific survival. The Cox regression analysis showed stage, grade and TIMP-2 to be significant predictors of survival. We suggest that TIMP-2 may have a more important role in endometrial carcinoma progression than TIMP-1 and might serve as a potential marker for favorable prognosis in this type of cancer.

Keywords TIMP-2 · Endometrial carcinoma · Prognosis · Invasion

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Introduction

Endometrial carcinoma is the most common invasive malignancy of the female genital tract in the Western world [1]. Annually, endometrial carcinoma develops in nearly 200,000 women worldwide, and an estimated 50,000 women die of this carcinoma [2]. In Finland, there were 809 new cases in 2009, and the incidence is expected to continue rising [3]. Endometrial carcinomas can be designated as type I or type II. Type I tumors, consisting mainly of endometrioid adenocarcinomas and accounting for approximately 80% of all endometrial carcinomas, are believed to develop in an estrogen-related manner. These tumors tend to be of low grade and well differentiated, thus carrying a better prognosis. Type II tumors, consisting mostly of serous and clear cell carcinomas, follow the estrogen-unrelated pathway and arise in the background of the endometrium. These tumors are typically of high grade and poorly differentiated and are characterized by a more aggressive clinical course and poorer prognosis than type I tumors [4, 5].

Tumor metastasis is a multistep process by which a subset or individual cancer cells disseminate from a primary tumor to distant secondary organ or tissues [6]. This process is facilitated by the tumor cells' ability to degrade the extracellular matrix (ECM), including the basement membrane. Matrix metalloproteinases (MMPs) are a large family of proteolytic enzymes playing key roles in virtually all events of ECM remodeling and turnover [7]. They principally degrade the ECM components, but have also other substrates such as cytokines, growth factor receptors, cell–cell, and cell-matrix adhesion molecules which may also take part in the invasion process [8, 9].

Tissue inhibitors of metalloproteinases (TIMPs) are considered some of the most important regulators of metalloproteinase activity. To date, four TIMPs have been characterized in humans, designated as TIMP-1, -2, -3, and -4. TIMPs are natural inhibitors of MMPs. In addition to their inhibitory role, TIMPs also possess growth-promoting capacities and can act as regulators of angiogenesis and programmed cell death. Their role in tumor invasion and spread is therefore controversial [10].

Only a few studies have been published concerning the role of tissue inhibitors of metalloproteinases -1 and -2 in endometrial carcinoma [11–14]. The present study aimed to evaluate the frequencies of TIMP-1 and -2 and their impacts on survival as well as their associations with conventional prognostic markers in a large patient material with a long follow-up.

Materials and methods

The patient material consisted of 241 patients diagnosed with endometrial cancer who underwent surgical treatment at Oulu University Hospital during 1992–2000. TIMP-1 protein was immunostained in the tumor sections of 230 patients, TIMP-2 for 241 patients. Because of the limited amount of tumor sections available in some cases, we were not able to stain 11 cases for TIMP-1. The median age of the patients was 65 years (range 37–98). Their median body mass index at the time of the diagnosis was 29 kg/m^2 (range 19–49). According to the FIGO criteria, there were 160 stage I, 35 stage II, 40 stage III, and 6 stage IV cancers. Of the material, 122 tumors were well (grade 1), 83 moderately (grade 2), and 36 poorly (grade 3) differentiated. Out of the 241 patients, 228 had endometrioid adenocarcinoma. The other cases were three adenoacanthomas, two adenosquamous carcinomas, six serous papillary carcinomas, and two clear cell carcinomas. The median follow-up time was 77 months (range 0–136).

In most cases, the primary treatments were extrafascial hysterectomy, bilateral salpingo-oophorectomy, and pelvic lymphadenectomy. Five patients had preoperative chemotherapy and 53 patients postoperative adjuvant cisplatin-based chemotherapy. Postoperative vaginal cuff brachytherapy was given to 31 patients and 95 patients received postoperative external whole pelvic irradiation. Both internal and external radiation therapy was given to 31 patients. Two patients received preoperative radiation therapy. Formalin-fixed, paraffin-embedded endometrial tissue samples from the primary tumors were obtained from the files of the Department of Pathology.

Immunohistochemical staining

Paraffin-embedded sections (4 μm) from the primary tumors of endometrial carcinoma were stained using the avidin–biotin–immunoperoxidase technique. Each tumor specimen was stained for both TIMP-1 (NCL-TIMP-1-485, Novocastra, Newcastle upon Tyne, UK) and TIMP-2 (MAB 971, R&D Systems, Minneapolis, MN, USA). The paraffin sections were first incubated overnight at 37°C, deparaffinized in a histological clearing agent, Histo-Clear (National Diagnostics, Atlanta, GA, USA) and hydrated in descending alcohol series. Epitope retrieval was performed by microwaving the slides with 10 mM citrate buffer at pH 6 for 10 min and then cooling for 20 min at room temperature. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide in distilled water for 5 min. Non-specific binding was blocked by using Antibody Diluent (DakoCytomaton, Inc., Glostrup, Denmark) when diluting the primary TIMP-1 or TIMP-2 antibody. Antibodies were diluted 1:75 (TIMP-1) and 15 g/mL (TIMP-2) and the specimens were incubated for one hour in a humidity chamber at room temperature.

Immunohistochemical staining was continued using the LSAB2 System-HRP kit according to the manufacturer's instructions. Biotinylated link antibody and peroxidase-labeled streptavidin incubations were 10 min each. The slides were washed thoroughly with phosphate-buffered saline after each stage of the procedure. The antibody reaction was visualized by using a fresh substrate solution containing DAB, 3-3′diaminobenzidine. The sections were counterstained with Mayer's hematoxylin (Reagena, Toivola, Finland), dehydrated and mounted with Histomount (National Diagnostics, New Jersey, USA).

Evaluation of TIMP-1 and TIMP-2 immunostaining

The samples were evaluated by two independent observers blinded from the clinical data. The samples were considered as negative or positive according to the absence or presence of immunoreaction for TIMP-1 or -2 protein in the cytoplasm of the tumor cells. Depending on the extent of staining, the sections were scored as 0–3. The case was considered positive when >1% of the neoplastic cells

showed positive staining. Weak positivity was marked as + (1% <tumor cells with positive reaction ≤25%) and moderate positivity was ranked as ++ (25% <tumor cells with positive immunoreaction ≤50%). The staining was considered intensive when more than 50% (+++) of the neoplastic cells showed a positive reaction for TIMP-1 or -2.

Statistical analysis

All statistical analyses were carried out using the SPSS software system (v. 16.0) for Windows. The relationships between the clinicopathological categorical variables and TIMP-1 or TIMP-2 immunostaining were assessed with Fisher's exact test. For continuous variables, the Mann–Whitney *U* test was used. The cancer-specific 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. Cox regression model was used in multivariate analysis to assess the independency of the prognostic variables. Survival was defined as the time from primary operation to the date of death or last control visit. *p* values less than 0.05 were considered statistically significant.

Results

Intracytoplasmic staining for TIMP-1 and TIMP-2 immunoreactive protein in tumor cells was found in 202 out of 230 (88%) cases (Fig. 1) and 207 out of 241 (86%), respectively (Fig. 2, Table 1). There were no cell types in stroma that would have stained systemically. Intensive TIMP-1 staining was noted in 53 samples (23%), and 54 samples (22%) were intensively stained for TIMP-2. Table 2 shows that >25% tumor cells with positive (moderate and intensive) immunoreactions for TIMP-2 was seen only in endometrioid adenocarcinomas. In adenoacanthoma, adenosquamous, serous papillary, and clear cell carcinomas only ≤25% tumor cells showed positive staining for TIMP-2 (Table 2). The number of histologies other than endometrioid adenocarcinoma was too small for detailed analyses.

For survival analyses, the study population was divided in two groups based on the intensity of staining. The case was considered positive when >25% tumor cells showed positive immunoreaction. The survival analysis showed a statistically significant correlation between tumor immunoreactive protein expression of TIMP-2 and cancer-specific survival (p=0.041; Fig. 3a). Out of 241 patients, 39 **(**16%**)** died of endometrial carcinoma during the follow-up. Ten out of 99 patients (10%) presenting with TIMP-2 positive immunostaining died of the disease. The corresponding figure for the patients presenting with TIMP-2 negative staining was 29 out of 142 (20%). The Kaplan–Meier analysis showed that the 5-year cancer-specific survival rate of the patients with TIMP-2 positive immunostaining was 89% and that of the TIMP-2 negative patients 78%. A statistically significant correlation was also found between overall survival and TIMP-2 (p=0.036). In endometrial adenocarcinoma patients (n=228), a trend for a more favorable survival was observed in Kaplan–Meier analysis (p=0.069; Fig. 3b). Furthermore, when stage IA carcinomas were excluded (n=

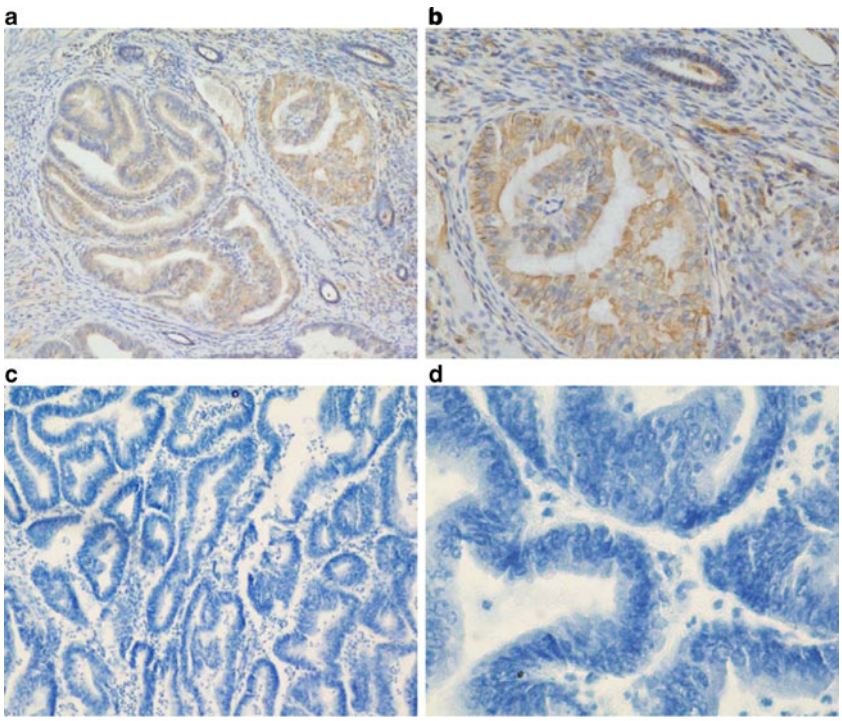

Fig. 1 Positive and negative cytoplasmic immunostaining for TIMP-1 in endometrioid adenonocarcinoma. **a** Positive, magnification ×70. **b** Positive, magnification ×350. **c** Negative, magnification ×70. **d** Negative, magnification ×350

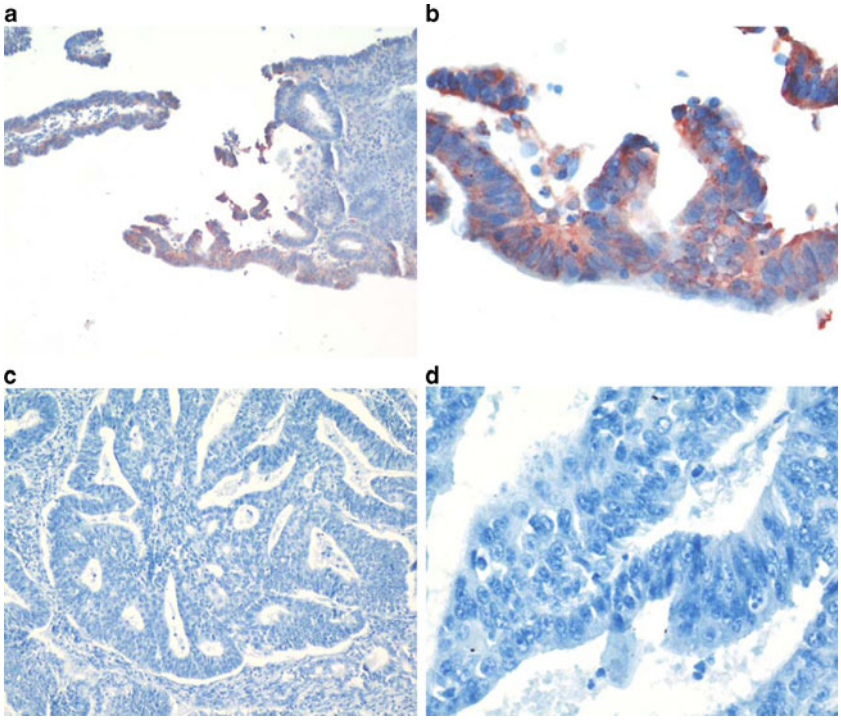

Fig. 2 Positive and negative cytoplasmic immunostaining for TIMP-2 in endometrioid adenonocarcinoma. **a** Positive, magnification ×70. **b** Positive, magnification ×350. **c** Negative, magnification ×70. **d** Negative, magnification ×350

198), the survival analysis showed statistically significant correlation (p=0.034; Fig. 3c). For TIMP-1, there was no statistically significant association with the overall or cancer-specific survival (data not shown).

The difference in cancer-specific survival between TIMP-2 negative and positive patients was seen after 2 years of follow-up (Fig. 3). The survival curves show that the difference increased until approximately 5 years and remained the same until the end of the follow-up time.

We could not find any correlation between the overexpression of TIMP-1 or TIMP-2 immunoreactive proteins and the stage of the disease or the histological grade of the tumor. Neither was there any correlation between patients' age, chemotherapy or radiation therapy and the positive immunoreaction for TIMP-1 or TIMP-2 in the primary tumor.

Using the Cox regression model, multivariate analysis of the following prognostic indicators was carried out: age, clinical stage, grade of histological differentiation, and TIMP-2 immunoreaction (Table 3). The Cox regression multivariate analysis showed stage (p< 0.001), grade (p< 0.001), and TIMP-2 (p= 0.054) to be significant predictors of survival. In endometrial adenocarcinoma patients, stage IA carcinomas excluded (n= 198), appeared stage (p=0.02), grade (p=0.006), and TIMP-2 (p=0.057) significant in the Cox multivariate analysis.

Table 1 TIMP-2 immunostaining according to patient characteristics (n=241)

Patient characteristics	Immunostaining				Total
	–	+	++	+++	
Stage					
I	26 (16)	70 (44)	30 (19)	34 (21)	160 (100)
II	4 (11)	14 (40)	8 (23)	9 (26)	35 (100)
III	4 (10)	21 (53)	6 (15)	9 (23)	40 (100)
IV	0	3 (50)	1 (17)	2 (33)	6 (100)
Grade					
1	14 (12)	55 (45)	21 (17)	32 (26)	122 (100)
2	15 (18)	34 (41)	17 (21)	17 (21)	83 (100)
3	5 (14)	19 (53)	7 (19)	5 (14)	36 (100)
Radiotherapy[a]	21 (13)	69 (43)	32 (20)	37 (23)	159 (100)
Chemotherapy[b]	11 (19)	30 (52)	9 (16)	8 (14)	58 (100)

Values are expressed as n (%)

– ≤1% tumor cells with positive reaction, + 1% <tumor cells with positive reaction ≤25%, ++ 25% <tumor cells with positive immunoreaction ≤50%, +++ >50% tumor cells with positive immunoreaction

[a] Brachytherapy, external radiotherapy or both

[b] Cisplatin-based chemotherapy

Discussion

In this study, we show for the first time an association between positive TIMP-2 immunoreactive protein expression and patients' favorable outcome in endometrial carcinoma. Results from previous studies concerning the relationship between TIMP-2 expression and patients' prognosis in other cancer types are conflicting. In breast cancer and in pancreatic ductal adenocarcinoma, high TIMP-2 protein expression was associated with better survival [15, 16]. In contrast to these

Table 2 TIMP-2 immunostaining according to histology (n=241)

Histology	Immunostaining				Total
	–	+	++	+++	
Endometrioid adenocarcinoma	29 (13)	100 (44)	45 (20)	54 (24)	228 (100)
Adenoacanthoma	2 (67)	1 (33)	0	0	3 (100)
Adenosquamous carcinoma	0	2 (100)	0	0	2 (100)
Serous papillary carcinoma	2 (33)	4 (67)	0	0	6 (100)
Clear cell carcinoma	1 (50)	1 (50)	0	0	2 (100)

Values are expressed as n (%)

− ≤1% tumor cells with positive reaction, + 1% <tumor cells with positive reaction ≤25%, ++ 25% <tumor cells with positive immunoreaction ≤50%, +++ >50% tumor cells with positive immunoreaction

studies, in bladder cancer a statistically significant association between TIMP-2 and poor survival was found, but both of these studies were performed in a relatively small number of patients [17, 18].

In our current study, TIMP-2 protein expression in cancer cells was associated with favorable cancer-specific and overall survival. This result is in line with the main function of TIMP-2, i.e., the inhibition of MMP-2, and with the findings derived from our previous research [19]. High expression levels of MMPs have previously been shown to correlate with poor prognosis in different human cancer types. In endometrial carcinoma, previous studies suggest that MMP-2 is the main metalloproteinase involved in the malignant behavior of endometrial cancer [20, 21].

An advantage of our study is that the patients were followed up systematically after the operation and the follow-up time was long. The difference in cancer-specific survival was seen only after 2 years of follow-up (Fig. 3), suggesting that long follow-up time is very significant in this type of cancer. The difference in survival increased until approximately 5 years and remained the same until the end of the follow-up time. The majority of adjuvant therapies are given during the first 2 years after diagnosis. This could partly explain why almost the entire study group survived the first years in a similar manner, the difference in survival not being seen until approximately 2 years. Our results suggest that TIMP-2 may help in identifying the patients with more aggressive disease at the time of the diagnosis. The patients with high TIMP-2 immunoreactivity might benefit from close follow-up, especially during the first 2 years after surgery. It also suggests that TIMP-2 could add some value as a prognostic marker in deciding about adjuvant therapies.

TIMP-2 is a soluble unglycosylated protein with a molecular mass of 21 kDa, which shares approximately 40% sequence identity with TIMP-1 [10, 22]. Low concentrations of TIMP-2 have been associated with MMP-2 activation and high concentrations with MMP-2 inhibition [23, 24]. TIMP-2, unlike TIMP-1, is also an effective inhibitor of the membrane-type MMPs [25]. TIMP-2 expression is constitutive, whereas external stimuli such as growth factors, phorbol esters, serum, and cytokines induce TIMP-1 expression in various cell types [23].

Invasion is a key event in cancer cell progression. Previous studies have suggested that the balance between active metalloproteinase and inhibitor is a critical determinant of tumor cell invasiveness. We found that when including only the subgroup of endometrioid adenocarcinomas for survival analyses, the difference did not quite reach statistical significance. When excluding the superficial stage IA carcinomas, the survival analyses again showed statistically significant correlation. Our present findings suggest that TIMP-2 inhibits tumor cell invasion in endometrial carcinoma. It supports the theory that TIMP-2 inhibits MMP-2 in infiltrating endometrial carcinoma. This finding is significant, because TIMP-2 is found as a complex with MMP-2, an enzyme that is closely linked with tumor cell invasion.

TIMP-1 is a soluble, glycosylated protein with a molecular weight of 28 kDa [10]. It is expressed by a variety of cultured cell types including fibroblasts, epithelial and endothelial cells, osteoblasts, chondrocytes, smooth muscle cells and many tumor cells [26]. TIMP-1 inhibits MMP-9, but it is not able to interact with MMP-2, because TIMP-1 lacks the critical C-terminal MMP2-interacting residues that are present in TIMP-2 [27].

Most of the existing literature on TIMPs concentrates on the MMP inhibitory function. Previously TIMPs' role as MMP inhibitors seemed most important, and there is evidence that downregulation of TIMP-1 and -2 expressions is associated with increased invasiveness of tumors, while overexpression leads to reduced tumor growth and metastasis formation [25, 28]. However, during recent years their multifunctional, and partly controversial, actions have come to be more and more appreciated. Their role in tumor progression and invasion is therefore far from clear. The new emerging concept is that TIMPs function in a contextual

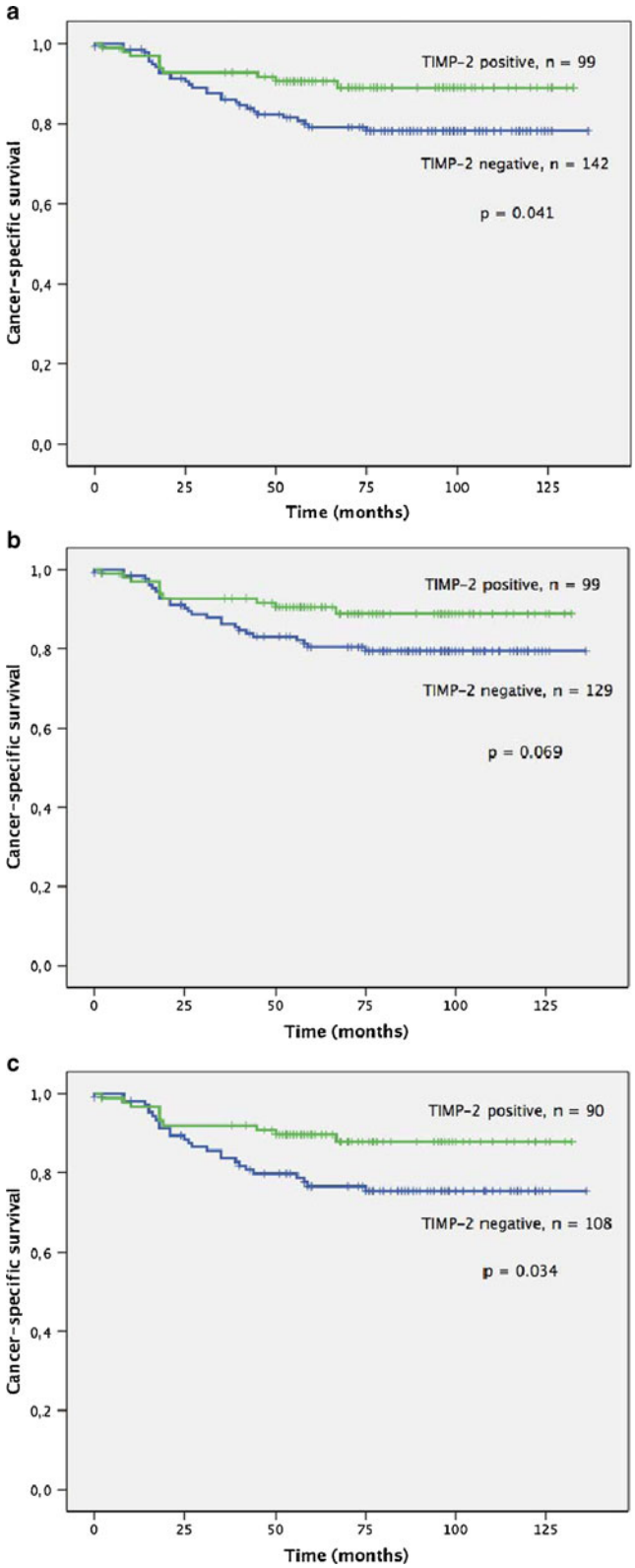

Fig. 3 Kaplan–Meier cancer-specific survival curves for the endometrial carcinoma patients according to the TIMP-2 immunoreactivity of the primary tumor. **a** Cancer-specific survival rate of all patients (n=241). **b** Cancer-specific survival rate of endometrioid adenocarcinoma patients (n=228). **c** Cancer-specific survival rate of endometrioid adenocarcinoma patients, excluded stage IA (n=198)

Table 3 The independent and significant prognostic factors for the cancer-specific survival of endometrial cancer patients as evaluated by the Cox regression method

Variable	Relative risk of death	p value to remove
Stage		<0.001
I	1	
II	2.80 (1.16–6.74)	
III	3.30 (1.51–7.21)	
IV	18.11 (5.65–58.07)	
Grade		0.001
1	1	
2	3.16 (1.38–7.26)	
3	4.93 (1.97–12.33)	
TIMP-2		0.054
Negative	1	
Positive	3.70 (0.96–4.15)	

Relative risk of death, its 95% confidence interval and p value are given for each covariate, n=241; did not significantly improve the model p value to enter, age 0.271

fashion so that their mechanism of action depends on the tissue microenvironment [29].

In our present work, we did not find any correlations between TIMP-1 or -2 immunostaining and conventional prognostic indicators of endometrial cancer. In a study by Graesslin et al. [13], histological grade correlated with TIMP-2 expression in endometrial cancer, but the number of patients was small (n=38). We do not know the reason for the difference in our results, because we could not obtain corresponding significance even with large patient material.

In conclusion, we show here that positivity for TIMP-2 immunoreactive protein in endometrial tumors has a direct positive influence on patients' survival. These data are in agreement with the primary action of TIMP-2: the inhibition of tumor invasion by inhibiting MMP-2. In contrast, tissue TIMP-1 does not yield useful clinical data. TIMP-2 seems to be an important regulator of cancer progression, especially invasion, but its multiple functions and the conflicting data reported need to be elucidated by further studies.

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Conflict of interests None.

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