

Serum tissue inhibitor of metalloproteinase 1 (TIMP-1) and vascular endothelial growth factor A (VEGF-A) are associated with prognosis in esophageal cancer patients

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ABSTRACT

Purpose: The matrix metalloproteinases, tissue inhibitors of metalloproteinases and angiogenesis contribute to growth and spread of cancer. We investigated the correlation between pretreatment serum levels of tissue inhibitor of metalloproteinase 1 (TIMP-1) and vascular endothelial growth factor A (VEGF-A), and clinicopathologic features and survival in patients with esophageal cancer (EC).

Material/Methods: Serum TIMP-1 and VEGF-A were measured by enzyme-linked immunosorbent assay (ELISA) in 89 patients with EC, and 30 healthy controls.

Results: Serum TIMP-1 and VEGF-A levels were significantly higher in patients with esophageal carcinoma than in the control group ($p=0.001$ and $p<0.001$, respectively). High levels of TIMP-1 were associated with histological type ($p<0.001$), tumor depth ($p<0.001$), stage ($p<0.001$) and lymph node metastases ($p=0.001$). Subgroup analysis showed that tumor size ($p<0.001$), tumor depth ($p<0.001$), stage ($p<0.001$), lymph node metastases ($p=0.002$), distant metastases ($p=0.009$) and resectability ($p=0.003$), were correlated with an elevated level of VEGF-A. Patients with elevated levels of TIMP-1 and VEGF-A had a significantly lower overall survival ($p=0.02$ and $p=0.048$, respectively), and disease-free survival (TIMP-1, $p<0.001$).

Conclusion: High serum levels of TIMP-1 and VEGF-A were found to be associated with tumor progression and unfavorable prognosis in patients with EC.

Key words: esophageal cancer, serum TIMP-1, serum VEGF-A, prognosis

INTRODUCTION

Esophageal cancer (EC) is one of the most aggressive and lethal malignancies [1]. In unselected patients, the overall 5-year survival rate is less than 10%, largely because of late disease presentation. Early lymphatic spread into the regional lymph node and relapse after surgical resection are a special feature of EC. In particular, the presence of lymph

node metastasis and vascular invasion indicates the highly malignant potential of EC. The invasion and metastatic capacity of EC cells is related to their ability to degrade the extracellular matrix and components of the basement membrane [2,3].

The spread of malignant tumors is a multistep process involving rapid growth and invasion into lymphatics and blood vessels. Structural changes in the extracellular

matrix are necessary for cell migration during tissue remodeling and tumor invasion. The regulated proteolysis of the extracellular matrix required for endothelial cell proliferation, migration and invasion is principally mediated by the activity of members of the matrix metalloproteinase family. Matrix metalloproteinases can collectively degrade all components of the extracellular matrix, and their requirement during angiogenesis has been demonstrated in matrix metalloproteinase-deficient mice [4,5]. Several distinct classes of proteins have been found to inhibit matrix metalloproteinases activity and thereby modulate the angiogenic process. Tissue inhibitors of metalloproteinases (TIMPs) are well-studied inhibitors of metalloproteinases and consist of a family of four structurally related proteins (TIMP-1-4) [6]. The TIMPs inhibit metalloproteinase activity associated with tumor invasion, angiogenesis and it is now widely appreciated that TIMPs have direct effects on cellular behaviors such as cell growth, apoptosis, migration and differentiation [7]. TIMP-1 has been shown to inhibit angiogenesis, endothelial cell migration, but not proliferation via a mechanism that involves direct inhibition of metalloproteinase activity [8]. Increased expression of matrix metalloproteinases and its tissue inhibitors is related to tumor aggressiveness and overall survival in several human tumors [9,10]. The use of secreted metalloproteinases and TIMPs as a serum marker of malignant cancer has also been evaluated [11-16].

Angiogenesis is an invasive process that requires proteolysis and remodeling of the extracellular matrix, the proliferation and migration of endothelial cells, as well as the synthesis of new matrix components [17,18]. Vascular endothelial growth factor A (VEGF-A) is one of the most potent angiogenic factors in solid tumors and it plays an important role in the hematogenous spread of the tumor [19]. The tissue expression of VEGF-A in EC has been associated with the depth of tumor invasion, the presence of local lymph node metastases, the presence of distant metastases, and patient outcome [2,20,21]. It has been reported that high levels of VEGF-A have been found in serum of patients with EC, and it has been correlated with tumor size, tumor stage, nodal status, distant metastases and poor survival [22-24], although not in all studies [25].

In the current study, we measured the circulating TIMP-1 and VEGF-A in the serum of patients with primary EC, and compared these levels to those in healthy subjects. We correlated the concentration of the examined factors with clinicopathological findings and survival. In addition, we defined the diagnostic sensitivity, specificity, and areas under receiver-operating characteristics (ROC) curves (referred to as AUCs) for the measurands and potential clinical use of serum VEGF-A and TIMP-1 as biomarkers of EC.

MATERIALS AND METHODS

Patients

Serum samples were obtained from 89 individuals: 63 patients with histopathologically confirmed squamous cell carcinoma of the esophagus and 26 with adenocarcinoma of the lower part of the esophagus or cardia treated in the Department of Thoracic Surgery of the Medical University of Białystok. Among the cancer patients, there were 9 females and 80 males, median age 62.0 years (range:36-83). All primary tumor cases were staged clinically according to the guidelines of the TNM staging system (7th edition) [26] system on the basis of the upper digestive tract: contrast radiographic studies of the upper digestive tract with barium or gastrografin, endoscopy with biopsy and pathologic examination, endoscopic ultrasound, posteroanterior and lateral chest radiography, ultrasound examination of the abdominal cavity and cervical nodes, thorax and abdominal cavity computed tomography (CT), as well as laparotomy and thoracotomy. Magnetic resonance imaging, bronchofiberscopy, endobronchial ultrasonography-directed fine-needle aspiration (EBUS-FNA), or positron emission tomography (PET/CT) scanning were additionally performed if indicated. The diagnosis was confirmed each time by microscopic examination of the material obtained during biopsy and/or surgery. In the study, the following physical and pathological factors were evaluated: age, sex, tumor location, tumor size, histological grading, the depth of tumor invasion (T factor), lymph node metastases (N factor), distant metastasis (M factor), stage, resectability and residual tumor. Fifty-four of the cancer patients underwent surgical tumor resection with lymphadenectomy. Thirteen patients underwent concurrent chemo-radiotherapy preoperatively, including cisplatin and 5-fluorouracil regimen associated with radiation (30 gray). Twenty-nine patients received chemotherapy, radiotherapy, or both postoperatively. Of the 89 cancer patients, 35 did not undergo esophagectomy. In most of the 35 cases, it was due to the advanced status of the disease, which was evidenced by the direct involvement of adjacent vital organs via local tumor extension (n=5) or the presence of distant organ metastasis (n=18); in others, it was due to poor performance status (n=10); and in some, it was due to the patients' refusal to undergo the operation (n=2). Of the 35 inoperable cases, 17 patients had undergone chemotherapy. Radiotherapy (52gray) was administered to 6 patients. A stenting procedure was performed in three patients. Double stenting (esophagus and trachea) was required in two patients. Six patients underwent feeding gastrostomy or jejunostomy and 2 patients refused further medication. After treatment, morbidity and mortality were documented. All the patients survived for at least 1 month after treatment and were followed up periodically, at intervals of 3-6 months, with clinical and laboratory examinations, including ultrasonography, CT and chest radiography. The median follow-up period was 17.5 months (range: 1-84

months). A total of 64 of the patients died of cancer during the 8-year follow-up. No patients had received any blood transfusion or undergone radiotherapy or chemotherapy prior to the start of the current study.

Controls subjects

The control group consisted of 30 healthy volunteers (24 men and 6 women). The median age of the controls at the time of sampling was 61.0 years (range: 33-77). There were no significant differences in age or gender between the patients and controls ($p=0.61$ and $p=0.15$, respectively).

Serum TIMP-1 and VEGF-A analysis

Venous blood samples were drawn into sterile vacuum tubes when esophageal carcinoma was detected before surgery or before anticancer therapy. To standardize clotting conditions, all sera were separated within 1 h after blood collection, aliquoted and stored at -80°C until assayed.

Serum levels of TIMP-1 and VEGF-A were measured using enzyme-linked immunosorbent assay kits (ELISA) (R&D Systems, Abingdon, England) according to the manufacturer's instructions. The serum samples were diluted 100-fold before determination. The manufacturer of assay kits referred to the intra-assay coefficient of variation (CV%) as 3.9% at TIMP-1 mean concentration of 1.27 ng/mL, $SD=0.05$ and as 6.7% at VEGF-A mean concentration of 53.7 pg/mL, $SD=3.6$. In the ROC report, the cut-off value corresponds to the highest accuracy (minimal false-negative and false-positive results).

Statistical analysis

Because the distributions of the TIMP-1 and VEGF-A showed no normal distributions (Shapiro-Wilk test), all studied groups were characterized by median values and as an interquartile range. The statistical differences regarding these variables in relation to several clinical and pathological parameters were assessed using the Mann-Whitney's U-test for two groups and the Kruskal-Wallis one-way analysis of variance (ANOVA) for three or more groups. Spearman's rank correlation analysis was used to describe the relationship between TIMP-1 and VEGF-A. The overall survival (OS) was defined as the time from treatment initiation (surgical resection, chemotherapy or radiation) to the date of death from any cause or the last known follow-up examination. Disease-free survival (DFS) was defined as the time from treatment initiation to the date of progression or the date of the last follow-up. Patients who died within 30 days of the operation were excluded from the study. The Kaplan-Meier method was used to estimate the probability of overall survival as a function of time. The differences in the survival of the subgroups of patients were compared using Mantel's log-rank test. The prognostic value of TIMP-1 and VEGF-A

was examined in univariate and multivariate analysis with the Cox's proportional hazard model. All p values were based on two-tailed statistical analysis, and a p value less than 0.05 was considered significant. Moreover, we calculated the diagnostic criteria, such as diagnostic sensitivity, specificity and AUCs for the measurands. Statistical analyses were carried out using the Statistica 8.0 PL program (StatSoft Inc., Tulsa, OK, USA). The diagnostic criteria and ROC curves were calculated using the ROC analysis package included in the Statistica 8.0 PL program.

In accordance with the declaration of Helsinki, the study protocol was approved by the local ethics committee (No R-1-002/103/2007) and written informed consent was obtained from all participants before analysis.

RESULTS

In EC patients' serum levels of TIMP-1 and VEGF-A were found to be statistically higher compared to control group ($p=0.001$ and $p<0.001$, respectively). The associations between the clinicopathological characteristics and the TIMP-1 and VEGF-A levels in patients with EC are summarized in *Tab. 1*. The level of TIMP-1 had no association with age, location, tumor size, histological grading, distant metastasis, resectability and residual tumor, but had a significant relationship with histological type, tumor depth, stage and lymph node metastasis. The VEGF-A level was associated with tumor size, tumor depth, stage, lymph node metastasis, distant metastasis, and resectability. Spearman rank correlation analysis revealed a significant positive relationship between serum TIMP-1 and VEGF-A levels in this group of EC patients ($R=0.217$, $p=0.039$).

Using ROC curves, sensitivity, specificity, positive and negative predictive values (PPV and NPV) were calculated for disease presence for each possible threshold value. The optimal cut-off value for application of the levels of TIMP-1 and VEGF-A as markers of disease presence were 180 ng ml^{-1} and 88 pg ml^{-1} , respectively. The diagnostic value of TIMP-1 and VEGF-A were: sensitivity – 80% and 83%, specificity – 60% and 70%, PPV – 85% and 89%, NPV – 50% and 60%, respectively. The AUC for TIMP-1 (0.734; 95% CI: 0.649-0.85) was lower than for VEGF-A (0.865; 95% CI: 0.8-0.93) $p=0.024$ (*Fig. 1*).

For assessing the prognostic value of each marker, we analyzed the patients with resected tumors. The median follow-up period operated patients was 33.5 months (range: 2- 84 months). Patients with TIMP-1 and VEGF-A levels less than the median levels (213 ng ml^{-1} and 136 pg ml^{-1}) were assigned to the low level groups, whereas those with levels higher than the median were assigned to the high level groups. For TIMP-1, the median OS of patients in the low level group was 43 months (95% confidence interval (CI): 26-55), and patients in the high level group was 25 months

Table 1. Correlation between serum TIMP-1 and VEGF-A levels and clinical and pathological characteristics of esophageal cancer.

Parameters	n	TIMP-1 (ng ml ⁻¹) Median, IQ ₁ -IQ ₃	P value	VEGF-A (pg ml ⁻¹) Median, IQ ₁ -IQ ₃	P value
Esophageal cancer	89	216(190-258)	0.001 ^a	175(98-323)	<0.001 ^a
Healthy	30	173(148-202)		50(20-98)	
Age					
<62	44	215(195-253)	0.78 ^a	168(87-278)	0.23 ^a
>=62	45	217(187-276)		178(126-351)	
Sex					
F	9	183 (173-222)	0.048 ^a	136(93-220)	0.36 ^a
M	80	217(195-262)		178(98-351)	
Location					
Upper	5	187(177-236)	0.1 ^b	559(215-559)	0.07 ^b
Midthoracic	41	226(184-255)		171(108-312)	
Lower	43	222(207-258)		155(78-351)	
Tumor size					
<4cm	25	207(170-227)	0.12 ^a	110(49-172)	<0.001 ^a
>=4cm	64	217(208-287)		215(136-414)	
Histological type					
Sqcc	63	228(208-278)	<0.001 ^a	181(98-312)	0.96 ^a
Adc	26	184(170-233)		159(96-331)	
Histological grading					
G1	13	205(173-217)	0.39 ^b	101(45-184)	0.053 ^b
G2	43	213(193-258)		180(133-351)	
G3	33	222(192-303)		203(93-382)	
Tumor depth					
T1+T2	20	190(144-207)	<0.001 ^a	95(45-164)	<0.001 ^a
T3+T4	69	234(210-284)		210(136-414)	
Stage					
I+II	26	190(170-219)	<0.001 ^a	126(49-172)	<0.001 ^a
III+IV	63	236(215-287)		220(138-414)	
N0	25	197(173-222)	0.001 ^a	118(52-180)	0.002 ^a
N1	64	235(213-287)		213(136-382)	
M0	71	214(185-258)	0.61 ^a	160(93-278)	0.009 ^a
M1	18	217(215-287)		312(211-457)	
Resectable	54	213(173-241)	0.4 ^a	136(78-264)	0.003 ^a
Nonresectable	35	217(209-288)		245(137-403)	
R0	46	207(173-241)	0.23 ^a	133(78-264)	0.2 ^a
R1+R2	8	229(203-243)		166(121-363)	

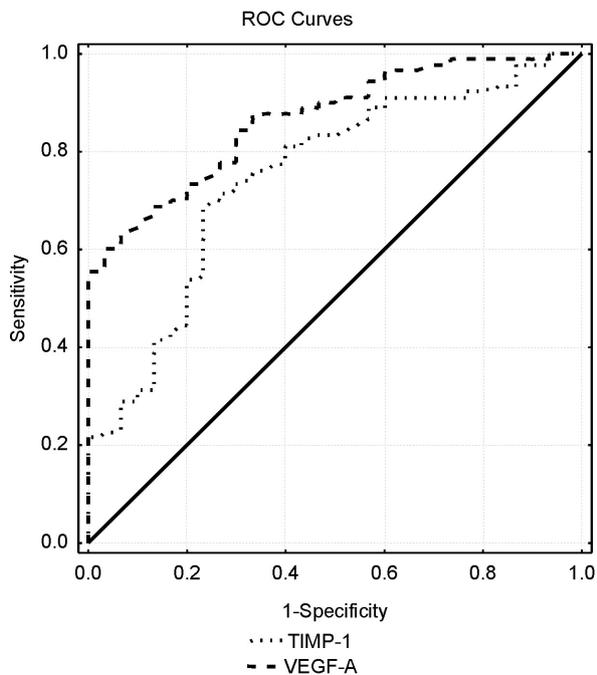
^aMann-Whitney U-test; ^bKruskal-Wallis test; IQ₁-IQ₃ - interquartile range

(95% CI: 18-40). For VEGF-A, the median OS of patients in the low level group was 41 months (95% CI: 30-53), and patients in the high level group was 23 months (95% CI: 12-39). The OS rates of patients with high pretreatment TIMP-1 and VEGF-A levels were significantly lower than those of patients with low TIMP-1 and VEGF-A (p=0.02 and p=0.048, respectively) (Fig. 2b and Fig. 3b). For TIMP-1, the median DFS of patients in the low level group was 40 months (95% CI: 21-52), and patients in the high level group was 19 months (95% CI: 14-33). For VEGF-A, the median DFS of patients in the low level group was 38 months (95% CI: 24-45), and patients in the high level group was 18 months (95% CI: 8-36). The DFS rates of patients with high pretreatment TIMP-1

levels were significantly lower than those of patients with low TIMP-1 levels (p<0.001) (Fig. 2a). No association between VEGF-A levels (less and higher than median) and DFS was found (Fig. 3a).

In univariate analysis, tumor size, tumor depth, stage, lymph node metastasis, residual tumor, TIMP-1 and VEGF-A were found to be significant prognostic factors for OS. Furthermore, TIMP-1 was also shown to be significant unfavorable predictor for DFS. In multivariate analysis, lymph node metastasis and residual tumor were identified as independent factors in patients' prognosis. However, neither TIMP-1 levels nor VEGF-A levels, was independently associated with patients' DFS and OS (Tab. 2).

Figure 1. The areas under the ROC curves for TIMP-1 (----) and VEGF-A (---) in esophageal cancer patients versus healthy subjects.



DISCUSSION

The aim of the present study was to analyze the association between pretreatment serum levels of TIMP-1 and VEGF-A and the clinicopathological parameters and outcome in patients with EC.

We observed a significant elevation of serum levels of TIMP-1 and VEGF-A in our patient population in comparison with healthy controls. Our findings were in accordance with the findings of Wallner et al. [22], Shimada et al. [23], Krzystek-Korpaczka et al. [25] in EC, and Tamura et al. [11] in non-small cell lung cancer results, which showed that patients with cancer had significantly higher concentrations of serum VEGF-A than healthy people. Mroczko et al. [14] provided evidence that patients with gastric cancer had higher serum TIMP-1 levels than healthy subjects. Similar results were reported for patients with various types of cancer [27-29].

We found that the diagnostic value of serum TIMP-1 and VEGF-A as a disease marker were: sensitivity–80% and 83%; specificity – 60% and 70%, respectively. This is higher than Shimada et al. [23], who calculated serum VEGF-A levels for application as a marker of disease presence to have a 79% sensitivity and a 48% specificity. The AUC for VEGF-A was higher than that for TIMP-1.

Figure 2. Postoperative disease-free survival (a) and overall survival (b) of patients with esophageal cancer in relation to pretreatment levels of TIMP-1.

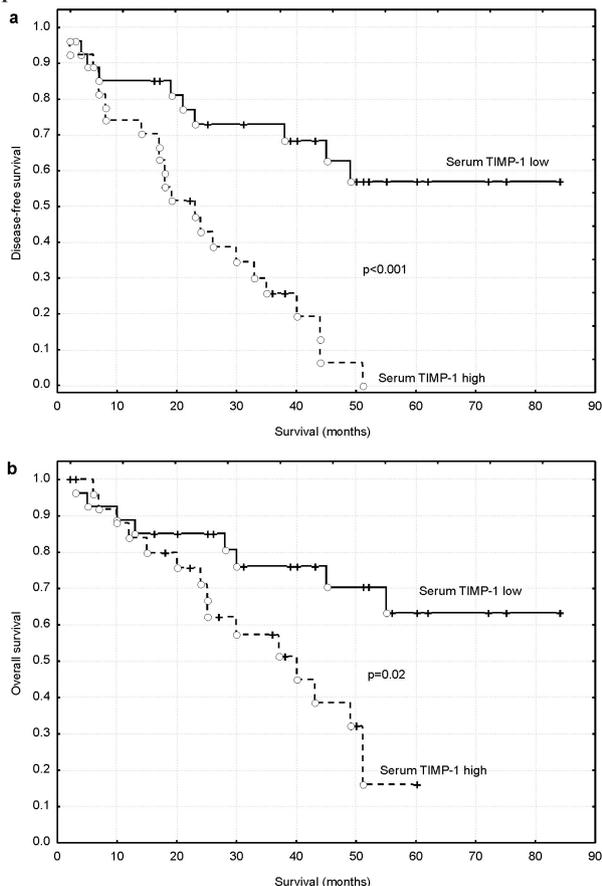


Figure 3. Postoperative disease-free survival (a) and overall survival (b) of patients with esophageal cancer in relation to pretreatment levels of VEGF-A.

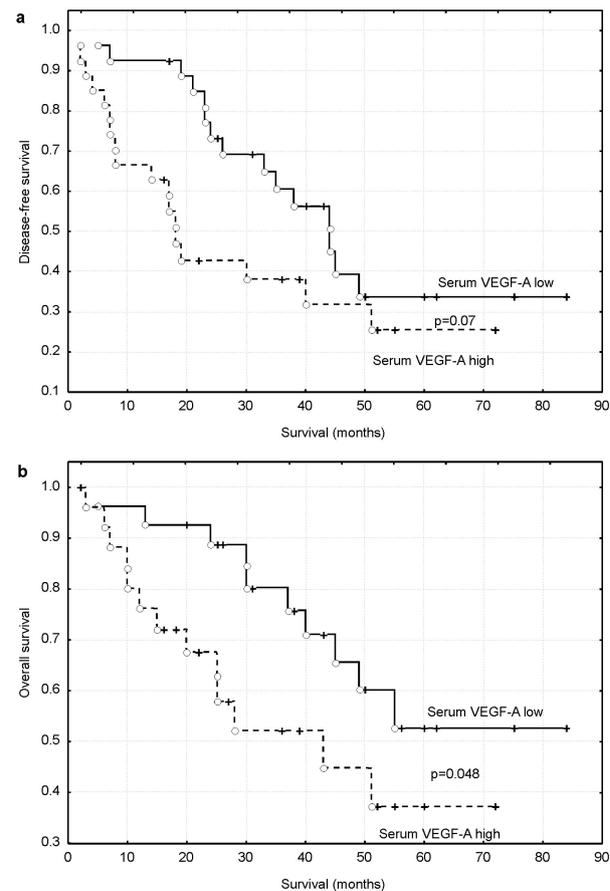


Table 2. Cox regression analysis of independent factors affecting disease-free and overall survival.

Factors	Disease-Free Survival				Overall Survival			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Sex	2.3(0.7-7.57)	0.17	-	-	4.63(0.62-34.44)	0.62	-	-
Location	1.04(0.48-2.24)	0.91	-	-	1.26(0.51-3.09)	0.61	-	-
Tumor size	3.54(1.69-7.42)	0.001	0.65(0.22-1.89)	0.42	3.51(1.43-8.6)	0.006	0.59(0.16-2.19)	0.43
Tumor depth T1+T2 vs. T3+T4	5.58(2.33-13.36)	<0.001	0.77(0.1-5.82)	0.8	5.58(1.95-15.97)	0.001	0.77(0.05-10.86)	0.85
Tumor stage I+II vs. III	6.87(3.13-15.07)	<0.001	2.06(0.21-19.83)	0.53	6.41(2.46-16.73)	<0.001	1.83(0.10-33.76)	0.86
Lymph node metastasis	8.95(3.58-22.39)	<0.001	7.42(1.7-32.22)	0.007	10.17(2.97-34.82)	<0.001	10.58(1.72-64.89)	0.011
Residual tumor R0 vs. R1+R2	4.99(2.2-11.29)	<0.001	7.5(2.44-23.05)	<0.001	9.86(3.59-27.04)	<0.001	14.15(3.96-50-53)	<0.001
TIMP-1	4.30(1.95-9.46)	<0.001	1.74(0.7-4.3)	0.23	2.96(1.21-7.22)	0.017	0.93(0.33-2.57)	0.88
VEGF-A	1.89(0.94-3.78)	0.078	-	-	2.11(0.91-4.85)	0.047	0.97(0.66-3.28)	0.71

In our investigation we demonstrated that circulating VEGF-A depended strongly on the spread of cancer cells beyond the mucosae and the esophagus. In this study, the levels of VEGF-A in EC were increased in tumors larger than 4 cm, deeper invaded lesions, the presence of lymph node metastases, distant metastases, advanced stages and non-resectable diseases. Our findings are in agreement with those obtained by Shimada et al. [23] who showed a significant correlation of VEGF-A with the tumor size, the depth of tumor invasion, N factor, M factor, and the stage of the disease. Nevertheless, Krzystek-Korpaczka et al. [25] could not establish any association between serum VEGF-A concentration and the clinicopathological parameters.

In gastric cancer, Mroczko et al. [14] demonstrated that serum TIMP-1 correlated significantly with the depth of tumor invasion and the presence of distant metastases. The same authors [28], proved that serum TIMP-1 in pancreatic cancer was positively associated with tumor depth, lymph node metastases, distant metastases, and non-resectable tumors. Mori et al. [30] have shown that TIMP-1 mRNA expression in squamous cell EC correlated significantly with lymphatic and venous invasion, the presence of lymph node metastasis, and advanced stage. In the current study, we found a significant association between serum concentration of TIMP-1 and histological type, tumor depth, lymph node metastasis and advanced stage. We did not find any correlation between serum levels of TIMP-1 and age, location, tumor size, histological grading, stage, distant metastases, resectability, and residual tumor. These findings suggest that serum levels of TIMP-1 may act as a positive factor for tumor progression and have an important role in esophageal tumorigenesis.

We demonstrated a significant correlation between pretreatment serum levels of TIMP-1 and postoperative

recurrence ($p < 0.001$). Patients with low serum TIMP-1 in the peripheral vein had a significantly longer DFS than patients with high serum concentration. Our findings are in agreement with those obtained by Wu et al. [27] in breast cancer, Rauvala et al. [31] in ovarian tumors, who showed a significant correlation of high TIMP-1 with shorter relapse-free survival. In our study, no association between VEGF-A levels in operated patients and DFS was found. We also found a significant correlation between pretreatment serum levels of TIMP-1 and VEGF-A and the OS in operated patients. Patients with high serum TIMP-1 and VEGF-A in the peripheral vein had a significantly shorter survival than patients with low serum concentration. Mori et al. [30] revealed that high TIMP-1 mRNA expression levels are associated with poorer OS of EC patients. Shimada et al. [23] showed that patients with high VEGF-A levels in the peripheral vein had shorter OS.

In the present study, tumor size, tumor depth, stage, lymph node metastases, residual tumor, TIMP-1 and VEGF-A levels had prognostic significance in the univariate Cox regression analysis. However, in multivariate analysis, serum TIMP-1 and VEGF-A levels were not found to be a significant independent prognostic factor. Uchida et al. [21] showed a significant difference in survival between VEGF-A positive overexpression and negative overexpression groups of EC patients. In their study, univariate analysis indicated that the depth of tumor invasion, lymph node metastases, distant metastases, and VEGF-A expression were prognostic determinants. Shih et al. [20] demonstrated that positive tumor overexpression of VEGF-A was a strong independent survival predictor in resected squamous cell EC. Mori et al. [30] revealed that high TIMP-1 mRNA expression level significantly correlated with survival, and it was an independent risk factor for overall survival in resected EC

patients. Our data are consistent with these studies and we suggest that the pretreatment serum TIMP-1 and VEGF-A levels might be useful for prognosis of patient survival.

TIMP-1 is an inhibitor of the matrix metalloproteinases, which may have a key role in cancer cell dissemination and endothelial cell migration in angiogenesis [32]. Recently, it has been demonstrated that TIMP-1 might be one of the factors involved in such a stimulation of proliferation and inhibition of apoptosis [33,34]. It has also been reported that TIMP-1 over-expression promotes tumor growth through stimulation of VEGF-A mediated activities [35]. In the present study, we observed a significant correlation between increased levels of TIMP-1 and VEGF-A. Moreover, we found high association between both TIMP-1 and VEGF-A levels, and tumor depth, advanced stage and lymph node metastasis. The unexpected association between high serum levels of TIMP-1 with early relapse and poor prognosis in EC seems to be a result of cancer promoting functions. We suggest that these observation may confirm the mitogenic and proangiogenic function of TIMP-1 in EC.

We found that TIMP-1 and VEGF-A levels exhibited a significant correlation with the tumor progression and prognosis. In locally advanced EC, we administered neoadjuvant chemoradiation therapy to improve resectability and local control, and adjuvant chemotherapy in the subgroup with node metastasis. Although judging resectability and selecting patients to combined modality therapy based on pretreatment serum TIMP-1 and VEGF-A levels is currently not practical (like other tumor markers), they may serve as a prognostic factors or perhaps a component of a prognostic index for EC.

CONCLUSIONS

In summary, our results suggest that TIMP-1 and VEGF-A play important role in dissemination of tumor cells and prognosis in patients with EC. Unfortunately, EC treatment is still far from perfect. The evaluation of TIMP-1 and VEGF-A in serum may therefore play important role in selecting EC patients for multimodality therapy, although this issue requires further investigation.

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