学　位　論　文

Stromal expression of cancer-associated fibroblast-related molecules, versican and lumican, is strongly associatedwith worse relapse-free and overall survival times in patients with esophageal squamous cell carcinoma

（食道扁平上皮癌間質でのCAF関連分子の発現は

予後に関連する）

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Abstract. Cancer-associated fibroblasts (CAFs) in the tumor microenvironment play an essential role in the tumor progression of esophageal squamous cell carcinoma (ESCC). The present study aimed to investigate the expression of CAF-related molecules, versican, periostin and lumican, in cancer stroma, to provide prognostic stratification for patients with ESCC after surgery. A total of 106 patients with ESCC who underwent curative esophagectomy without preoperative chemotherapy or radiotherapy were enrolled. The expression of CAF-related stromal proteins, including versican, periostin and lumican, was examined using immunohistochemistry, and the prognostic value was assessed by Kaplan-Meier survival analysis, and univariate and multivariate Cox regression models. The expression of versican, periostin and lumican was found specifically in the stromal component of ESCC. Kaplan-Meier analysis demonstrated that, compared with a low expression level, a high expression level of versican, periostin or lumican in the cancer stroma was significantly associated with worse relapse-free survival (RFS) and overall survival times in patients with ESCC. The prognostic values of stromal versican and lumican remained significant in a stratified analysis of stage I patients. Moreover, univariate and multivariate analysis revealed that high stromal versican or lumican expression was an independent prognostic factor for RFS in the patients. The present study demonstrated that CAF-related molecules, including versican, periostin and lumican, were expressed in the stroma of ESCC, and that stromal expression of versican and lumican in particular may have clinical utility as a prognostic biomarker for poor RFS in postoperative patients with ESCC.

Introduction

An estimated 572,034 new esophageal cancer cases and 508,585 associated deaths are expected annually according to the Global Cancer Statistics 2018 (1). Esophageal cancer is one of the least studied and most fatal cancer types worldwide due to its extremely aggressive nature and poor survival rate (2). Esophageal squamous cell carcinoma (ESCC) is the most common histological subtype of esophageal cancer in Asia, while adenocarcinoma is dominant in Western countries. Despite the global incidence of ESCC decreasing slightly in recent years, ESCC is still a major cause of cancer-related morbidity and mortality worldwide (3). The majority of patients with ESCC die due to local recurrence and distant metastasis, even after curative surgery; however, no prognostic biomarkers are currently used for the treatment decision in the clinical setting (4,5).

The tumor microenvironment (TME) is known to play an important role in esophageal cancer development and progression (6). The TME is composed not only of cellular components, such as cancer-associated fibroblasts (CAFs), endothelial cells and immune cells, but also of the extracellular matrix (ECM), a network of macromolecules that provide mechanical and biochemical support for surrounding cells (7). CAFs are commonly described as having a myofibroblastic phenotype; i.e., a secretory and contractile cell that expresses α-smooth muscle actin (αSMA)(6). CAFs regulate a number of tumor-promoting functions, including invasion and angiogenesis, and may also affect tumor cell function by remodeling and generating tissue tension (8). The increased expression of these CAF proteins is induced by growth factors and microRNAs secreted by cancer cells. CAFs can modulate tumor progression in several pathways, such as via the alteration of ECM protein structure and stiffness (6). In the TME, CAFs can produce ECM proteins, growth factors and cytokines to promote tumor progression and metastasis

(9). In a previous study, genome-wide expression profiling of ESCC demonstrated that CAF-related molecules, including versican, periostin and lumican, were highly expressed in ESCC (6). Versican, periostin and lumican are all TGF-β-related molecules in CAFs (6,10,11). Although the expression of all three molecules in cancer stroma was found to be associated with poor survival outcomes in several types of cancer and their tumor-promoting functions in the TME have also been reported (10,12-15), to the best of our knowledge, it remains to be determined whether the expression levels of these stromal proteins have a prognostic impact in ESCC. The present study aimed to address the prognostic role of CAF-related molecules, including versican, periostin and lumican, via immunohistochemical analysis of 106 specimens obtained from patients with stage I-IV ESCC treated by curative surgery without preoperative therapy.

Materials and methods

Patients and specimens.

From 303 consecutive patients with esophageal cancer who underwent curative esophagectomy between July 2004 and July 2019 at the Department of Gastrointestinal Tract Surgery (Fukushima Medical University, Fukushima, Japan), 106 patients with ESCC who did not receive preoperative chemotherapy or radiotherapy were enrolled in the present study (Fig. S1). The clinical and pathological data were retrospectively collected from medical records, with the date of last follow-up being July 2019. These data included age, sex, tumor location, tumor depth, presence of lymph node metastasis, lymphatic and venous invasion, and Tumor-Node-Metastasis (TNM) classification

defined by The TNM Classification of Malignant Tumors, 8th edition (16). This study was retrospective and the tissue samples for the patients were obtained from the Department of Gastrointestinal Tract Surgery and the Department of Diagnostic Pathology, Fukushima Medical University. This study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Fukushima Medical University.

Immunostaining and scoring.

For immunostaining, the EnVision system (Dako; Agilent Technologies, Inc.) was used to observe the expression of versican, periostin and lumican. Primary rabbit polyclonal antibodies, anti-versican (cat. no. HPA004726) and anti-lumican (cat. no. HPA001522), were purchased from Sigma-Aldrich; Merck KGaA, and rabbit polyclonal anti-periostin (RD181045050) was purchased from BioVendor R&D (10,12). Rabbit monoclonal anti-αSMA (cat. no. 19245) was purchased from Cell Signaling Technology, Inc. For immunostaining, the tissue samples were fixed in 10% formalin at room temperature for 48 h. and embedded in paraffin. The specimens were cut into 4-µm sections, which were deparaffinized in xylene and treated with a series of ethanol (100, 100 and 95% for 5 min each). Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol. For versican stainging, antigens were retrieved by autoclaving with 10 mM citrate buffer solution (pH 6.0) at 100˚C for 10 min. For the staining of periostin, lumican and αSMA, antigens were not retrieved. Next, the slides were incubated with the following primary antibodies: Versican (1:500), periostin (1:1,000), lumican (1:500), and αSMA (1:400). All primary antibody incubations were performed at 4˚C overnight. The sections were subsequently incubated with anti-rabbit secondary antibodies (DAKO Envisison+

System; cat. no. K4003; Agilent Technologies, Inc.). Peroxidase was visualized with diaminobenzidine (Dojindo), and the nuclei were counterstained with Mayer's Hematoxylin solution. Immunostaining score was composed of two factors: Staining intensity and percentage of positivity in the stromal area. Staining intensity was scored as follows: 0, negative; 1, weak; and 2, strong. The percentage of positivity in the stromal area was scored as follows: 0, 0-5%; 1, 5-25%; and 2, ≥25%. Scores were combined to generate each immunohistochemistry (IHC) score (min, 0; max, 4) (14). The individuals with a total score of 4 were defined as the high expression group, while individuals with a score of 0-3 were defined as the low expression group for each molecule. Evaluation of staining for αSMA was assessed as the percentage of positivity and the staining intensity in the stromal area; staining intensity was scored as follows: 0, negative; 1, weak; and 2, strong. The percentage of positive staining in the stromal area was scored as follows: 1, 0-50%; 2, >51%. The scores were combined to generate each IHC score (min, 0; max, 4). The individuals with a total score of 2-4 were defined as the high expression group, whereas individuals with total score of 0-1 were defined as the low expression group. Microscopic analysis was conducted using NanoZoomer-SQ (Hamamatsu Photonics K.K.) by three independent investigators, including two pathologists, who had been blinded to the clinical data, The scoring was determined through discussion.

Statistical analysis.

All statistical analyses were performed with R software (Ver. 3.6.1.) (17) in the present study. The χ^2 test was used to evaluate age, presence of lymph node metastasis, lymphatic invasion and venous invasion, and tumor location. Fisher's exact test was applied to analyze differences in sex, postoperative additional therapy and tumor differentiation, and

the Mann-Whitney U test was applied for tumor depth and the TNM classification for comparisons between each high and low group. Relapse-free survival (RFS) time was defined as the time from the date of surgery to the date of tumor relapse at any site, and overall survival (OS) time was defined as the time from the date of surgery to the date of death. RFS and OS were analyzed using the Kaplan-Meier method and log-rank and Wilcoxon tests. The associations between stromal versican, periostin, lumican and αSMA were calculated by the χ^2 test, and the association between stroma periostin and lumican calculated by Fisher's exact test. The Cox hazard regression model was used for univariate and multivariate survival analysis. The results were presented as hazard ratios (HRs) and their 95% confidence intervals (CIs). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of versican, periostin and lumican in ESCC as determined via immunohistochemistry.

The clinicopathological characteristics of the 106 patients with ESCC are summarized in Table SI. According to the expression of versican, periostin and lumican, as assessed by immunohistochemistry, the clinicopathological factors are shown in Table I. As demonstrated in Fig. 1A, versican staining was found specifically in the cancer stroma, and 50 out of 106 patients (47%) were determined to have high stromal versican expression. Likewise, the expression of periostin and lumican was also observed in the cancer stroma, and 66 (62%) and 23 (22%) tumors were

considered to exhibit high stromal periostin and lumican, respectively (Table I; Fig. 1B and C). The present study investigated whether cancer cells expressed the three CAF-related molecules, and the expression of periostin and lumican was detected, whereas that of versican was very low. The expression of periostin in cancer cells was significantly associated with that in stromal cells, while the expression of lumican in cancer cells did not exhibit any significant association with that in stromal cells (data not shown). By contrast, versican was not expressed in cancer cells, but was expressed in stromal cells. High levels of versican and periostin in the cancer stroma were significantly associated with aggressive clinicopathological features, including a greater depth of invasion, the presence of lymph node metastasis, positive lymphatic and venous invasion, and a more advanced TNM stage ($P<0.05$; Table I). In addition, high level of versican was associated with postoperative additional therapy, and high level of periostin was associated with location ($P<0.05$; Table I). High expression of stromal lumican was significantly associated with depth of invasion, lymph node metastasis, venous invasion and stage, but not with lymphatic invasion (Table I). As shown in Tables II-IV, a significant positive association was also found between stromal versican and periostin ($P<0.0001$), stromal periostin and lumican ($P<0.0002$), and stromal lumican and versican ($P=0.0034$). Moreover, the expression of all three molecules was positively associated with the expression of αSMA (Fig. S2 and Table SII).

High expression of stromal versican, periostin and lumican in ESCC tissues and the association with poor prognosis.

Kaplan-Meier survival analysis showed that patients with stage I-IV ESCC exhibiting high stromal versican expression experienced significantly shorter RFS and OS times, compared with those patients exhibiting low expression ($P<0.0001$ and $P=0.0003$, respectively; Fig. 1A). Similarly, stromal periostin and lumican expression demonstrated a significant impact resulting in poor prognosis for both RFS and OS (periostin, $P=0.0001$ and $P=0.0002$, respectively; lumican: $P<0.0001$ and $P=0.0004$, respectively; Fig. 1B and C). Moreover, when patients were stratified according to TNM stage, high expression of stromal versican and lumican each showed a significant association with poorer RFS compared with low expression only in stage I patients (Fig. S3). It was also found that the prognostic performance of stromal versican had statistically significant in the subgroup analyses of stage III patients. Similarly, stromal periostin tended to be associated with RFS in stage III patients, although this result was not significant.

Univariate and multivariate survival analysis for patient prognosis.

In the univariate survival analysis, RFS rate was associated with invasion depth (HR, 4.47; 95% CI, 2.04-9.82; $P<0.001$), lymph node metastasis (HR, 3.1; 95% CI, 1.44-6.71; $P=0.004$), lymphatic invasion (HR, 3.59; 95% CI, 1.57-8.22; $P=0.002$), venous invasion (HR, 9.26; 95% CI, 2.78-30.82; $P<0.001$), TNM stage (HR, 1.98; 95% CI, 1.42-2.75; $P<0.001$), stromal versican expression (HR, 9.11; 95% CI, 3.14-26.44; $P<0.0001$), stromal periostin expression (HR, 7.46; 95% CI, 2.24-24.88; $P=0.001$) and stromal lumican expression (HR, 5.11; 95% CI, 2.35-11.1; $P<0.0001$). Multivariate survival analysis by the Cox hazard model showed that three factors, TNM stage (HR, 1.81; 95% CI, 1.03-3.16; $P=0.039$), stromal versican expression (HR, 3.96; 95% CI, 1.16-13.46;

P=0.028) and stromal lumican expression (HR, 2.55; 95% CI, 1.06-6.17; P=0.037) were independent indicators for a poor prognosis (Table V).

Discussion

The present study investigated whether the expression of versican, periostin and lumican has utility as a prognostic biomarker for ESCC using 106 surgically resected specimens assessed via immunohistochemistry. The staining of the three CAF-related molecules in the cancer stroma was significantly associated with worse RFS and OS times. Moreover, stromal versican and lumican expression levels were independent prognostic factors for ESCC.

A previous study revealed that CAFs can increase the frequency of cancer stem cells, leading to a high tumor recurrence rate and a poor prognosis, which is enhanced by TGF-β signaling, while poor prognostic signatures share a stromal gene program that is induced by TGF-β (18). Another study showed that ovarian CAFs, which had much higher levels of TGF-β receptors than other cell types, exhibited versican upregulation by TGF-β. By contrast, TGF-β receptors were downregulated in ovarian cancer cells, possibly conferring resistance to inhibitory growth signals exerted by TGF-β (19). These results indicate that CAFs are specifically responsive to elevated TGF-β levels, while cancer cells can be the major source of TGF-β ligands (14). Furthermore, studies have shown that TGF-β signaling plays a role in esophageal cancer progression. For example, upregulation of TGF-β was associated with tumor size in patients with ESCC (20). Additionally, overexpression of TGF-β and decreased TGF-β receptor expression were associated with depth of invasion and pathological stage in patients with ESCC (21). TGF-β/Smad

signaling has been shown to promote epithelial-mesenchymal transition in ESCC (22,23). CAF-specific versican was upregulated by TGF-β in several cancer types, such as colorectal and ovarian cancer, resulting in cancer cell motility and invasion (14,19). Versican is implicated in the regulation of cell proliferation, differentiation, apoptosis, migration and adhesion in a variety of cancer types, such as breast and ovarian cancer (24). Versican is a large chondroitin sulfate proteoglycan that is a major component of the ECM (12,13) and plays a role in the formation of the tumor-specific ECM, which can support cancer cell growth and metastasis in certain solid cancer types. Several clinical studies indicated that high versican expression was a poor prognostic factor in a variety of cancer types such as prostate, breast and gastric cancer (24). The present study showed that high versican expression in the stroma was associated with poor RFS and OS times in stage I-IV ESCC after resection, which was consistent with earlier findings in other cancer types (25-28). Furthermore, the expression of stromal versican was significantly associated with poor RFS time in stratified analyses of stage I and III patients. Correspondingly, stromal versican was found to be an independent prognostic factor for RFS via multivariate analysis. These results indicated that stromal versican may be used as a prognostic biomarker for patients with ESCC after curative surgery, and that immunohistochemical analysis for versican expression in resected specimens may influence a decision on whether to complete intensive postoperative treatment, including administration of adjuvant chemotherapy, particularly for patients with stage I ESCC.

Periostin is an extracellular matrix secreted protein that is upregulated in tumor cells in several cancer types, including pancreatic, colorectal, lung, ovarian, breast, head and neck, thyroid, gastric, hepatic and esophageal cancer (5,15,29-36). Periostin overexpression in tumor cells, not in stroma, has also been associated with tumor invasion

and metastasis in oral carcinoma and esophageal cancer (37,38). Periostin is regulated by TGF-β signaling, as well as versican expression. An earlier study showed that periostin was expressed by fibroblasts in the normal tissue and in the stroma of the primary tumor (6). Infiltrating tumor cells need to induce stromal periostin expression in the secondary target organ to initiate colonization, and the induced periostin secreted by CAFs in the stroma of the metastatic loci was required to allow for the maintenance of cancer stem cells (24). Periostin is able to interact with other ECM proteins, specific cell surface receptors and integrins via multiple signal pathways affecting metastasis, invasion and angiogenesis in cancer development (39). Periostin was reported to bind as a ligand to αvβ3 and αvβ5 integrins, thus signaling via the PI3K-Akt pathway within esophageal cancer. The study reported that periostin-positive tumors exhibited higher levels of vascular endothelial growth factor and greater microvessel density compared with periostin-negative tumors (5). These findings indicate that periostin serves a key role in ESCC tumorigenesis through the induction and/or promotion of angiogenesis. Periostin is an important mediator of tumor invasion in ESCC (37). The present study showed that high stromal periostin was significantly associated with worse RFS and OS times.

Lumican is also known to be regulated by TGF-β signaling. Lumican in stromal tissues, adjacent to cancer cells, may modulate the characteristics of collagen fibers and induce the invasion activity of pancreatic cancer cells (40). A previous study showed that in breast cancer, a high expression level of stromal lumican was associated with a high pathological tumor grade (40). By contrast, a high expression level of lumican in breast cancer was reported to be associated with slow progression and an improved prognosis (41). In pancreatic cancer, lumican expression in cancer stroma is associated with a shorter survival time (42). Another study reported that the presence of lumican in the

ECM surrounding pancreatic ductal adenocarcinoma (PDAC) cells is associated with an improved patient outcome. Secretion of lumican from activated pancreatic stellate cells within PDAC is negatively regulated by TGF-β (11). In ESCC, to the best of our knowledge, there is still no report on lumican in association with prognosis, and there are few studies describing the molecular mechanisms of stromal lumican in malignant tumors (11). The present study showed that high stromal lumican expression indicated a poor prognosis in patients with ESCC, and a significant difference in RFS was found between high and low stromal lumican expression groups in the analysis of stage I patients. Furthermore, stromal lumican expression, as well as versican expression, was found to be an independent prognostic factor for RFS via multivariate analysis.

Overall, the present study examined the protein levels of versican, periostin and lumican via IHC without exploring the detailed molecular mechanisms. Further studies will be required to clarify the general role, mechanisms and relationships of versican, periostin and lumican in ESCC.

In summary, stromal periostin may have utility as a prognostic biomarker, while stromal versican and lumican, in particular, could be independent prognostic factors for ESCC. The results of the present study indicated that stromal versican and lumican expression scoring may help to make a decision on whether to administer adjuvant chemotherapy for patients with ESCC.

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Figure legends

Figure 1.

(A) Representative immunostaining for versican (low and high expression). High expression of stromal versican was significantly associated with worse RFS and OS times. (B) Representative immunostaining for periostin (low and high expression). High expression of stromal periostin was significantly associated with a worse prognosis compared with low expression in terms of RFS and OS times. (C) Representative immunostaining for lumican (low and high expression). High expression of lumican was found to be significantly associated with a poor prognosis

in terms of RFS and OS times. For stromal staining, the low group represents a score of 0-3, while the high group represents a score of 4. All survival data was assessed using the Kaplan-Meier method and log-rank test. Arrows indicate cellular staining. RFS, relapse-free survival; OS, overall survival.

Figure 1.

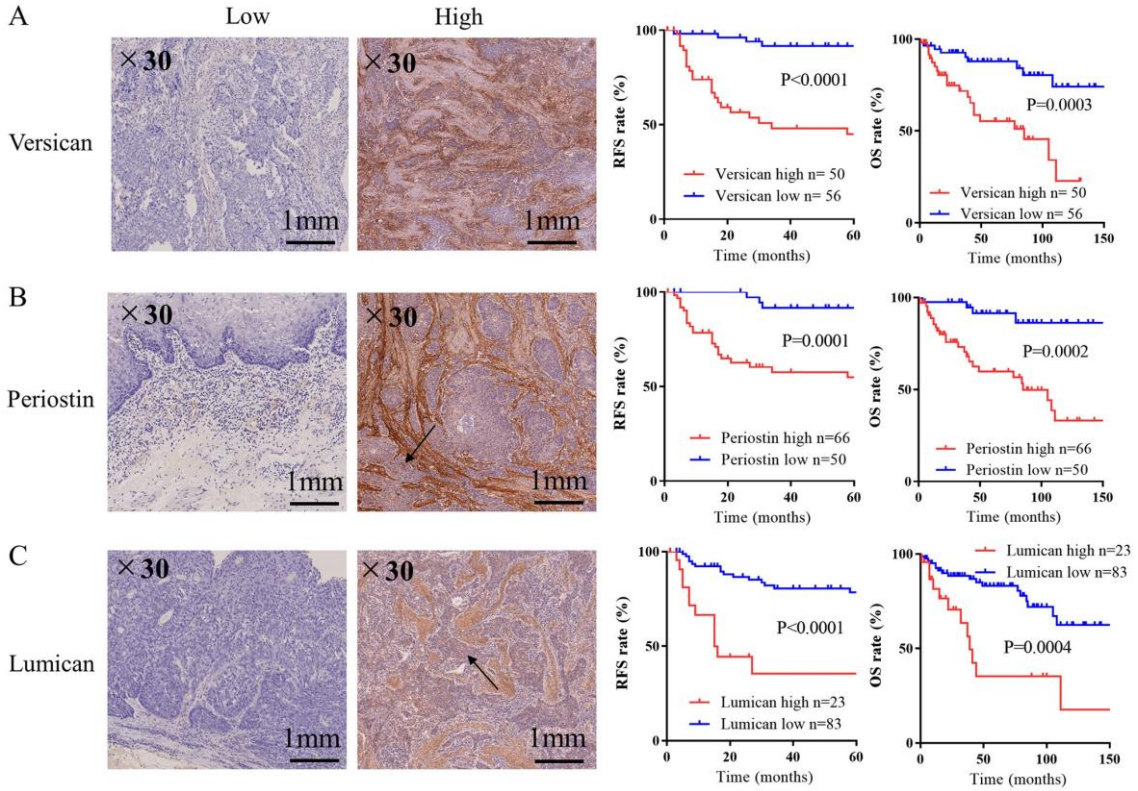
A
Low
High
Versican
×30
1mm
RFS rate (%)
P<0.0001
Versican high n= 50
Versican low n= 56
Time (months)
OS rate (%)
P=0.0003
Versican high n= 50
Versican low n= 56
Time (months)
B
Periostin
×30
1mm
RFS rate (%)
P=0.0001
Periostin high n=66
Periostin low n=50
Time (months)
OS rate (%)
P=0.0002
Periostin high n=66
Periostin low n=50
Time (months)
C
Lumican
×30
1mm
RFS rate (%)
P<0.0001
Lumican high n=23
Lumican low n=83
Time (months)
OS rate (%)
P=0.0004
Lumican high n=23
Lumican low n=83
Time (months)

Table I. Patient characteristics.

Characteristic	Versican expression			Periostin expression			Lumican expression		
	High (n=50)	Low (n=56)	P-value	High (n=66)	Low (n=40)	P-value	High (n=23)	Low (n=83)	P-value
Age, years									
Range (mean ± SD)	42-81 (65.82±8.55)	37-79 (64.00±8.53)	0.8011	42-81 (65.94±8.55)	37-78 (63.08±8.53)	0.1350	42-81 (66.26±8.55)	37-79 (64.47±8.58)	0.4588
≤60, n	14	18		16	16		5	27	
≥61, n	36	38		50	24		18	56	
Sex, n			0.3755			>0.999			0.4727
Male	42	51		58	35		19	74	
Female	8	5		8	5		4	9	
Postoperative additional therapy, n			0.0436[a]			0.0529			0.0569

No	36	50		49	37		17	69	
Chemotherapy	12	6		15	3		4	14	
Radiotherapy	2	0		2	0		2	0	
Chemoradiotherapy	0	0		0	0		0	0	
Location, n			0.2137			0.0409[a]			0.2884
Upper	4	11		5	10		1	14	
Middle	30	31		40	21		14	47	
Lower	16	14		21	9		8	22	
Invasion depth, n			<0.0001[a]			<0.0001[a]			0.0005[a]
pT1	25	49		34	40		9	65	
pT2	10	3		13	0		6	7	
pT3	14	4		18	0		8	10	

pT4	1	0		1	0		0	1	
Lymph node metastasis, n			0.0022[a]			0.0002[a]			0.0633
Yes	27	13		34	6		13	27	
No	23	43		32	34		10	56	
Lymphatic invasion, n			0.0002[a]			0.0049[a]			0.3773
Yes	33	16		38	11		13	36	
No	17	40		28	29		10	47	
Venous invasion, n			<0.0001[a]			0.0026[a]			0.0078[a]
Yes	40	17		46	11		18	39	
No	10	39		20	29		5	44	
Tumor differentiation, n			0.1365			0.8843			0.3300

Well	12	14		15	11		9	17	
Moderate	27	26		34	19		9	44	
Poor	10	8		12	6		3	15	
Unknown	1	8		5	4		2	7	
TNM stage, n			0.0075[a]			<0.0001[a]			0.0091[a]
I	25	42		30	36		8	59	
II	10	6		16	0		8	8	
III	11	7		15	3		7	11	
IV	4	1		4	1		0	5	

[a]P<0.05. TNM, Tumor-Node-Metastasis. Invasion depth (T stage) was determined according to the TNM classification of Malignant Tumors 8th edition (17).

Table II. Association between stromal versican and periostin expression levels.

		Periostin		P-value
		high (n=66)	low (n=40)	
Versican	high (n=50)	43	7	<0.0001
	low (n=56)	23	33	

Table III. Correlation between stromal periostin and lumican expression levels.

		Lumican		P-value
		high (n=23)	low (n=83)	
Periostin	high (n=66)	22	44	<0.0001
	low (n=40)	1	39	

Table IV. Correlation between stromal lumican and versican expression levels.

		Versican		P-value
		high (n=50)	low (n=56)	
Lumican	high (n=23)	17	6	0.0076
	low (n=83)	33	50	

Table V. Univariate and multivariate analysis for relapse-free survival.

Factor	Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (≤60 vs. ≥61 years)	1.00	0.45-2.22	0.9940			
Sex (male vs. female)	1.58	0.54-4.57	0.4027			
Invasion depth (pT1-2 vs. pT3-4)	4.47	2.04-9.82	0.0002[a]	0.80	0.30-2.15	0.6545
Lymph node metastasis (yes vs. no)	3.10	1.44-6.71	0.0040[a]	0.56	0.19-1.64	0.2870
Lymphatic invasion (yes vs. no)	3.59	1.57-8.22	0.0025[a]	1.01	0.37-2.79	0.9814
Venous invasion (yes vs. no)	9.26	2.78-30.82	0.0003[a]	3.07	0.78-12.09	0.1095
TNM stage	1.98	1.42-2.75	<0.0001[a]	1.81	1.03-3.16	0.0390[a]
Versican (high vs. low)	9.11	3.14-26.44	<0.0001[a]	3.96	1.16-13.46	0.0278[a]
Periostin (high vs. low)	7.46	2.24-24.88	0.0011[a]	1.91	0.48-7.65	0.3582

Lumican (high vs. low)	5.11	2.35-11.10	<0.0001[a]	2.55	1.06-6.17	0.0371[a]

[a]P<0.05. TNM, Tumor-Node-Metastasis; HR, hazard ratio; CI, confidence interval.