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

NAOTO YAMAUCHI1, YASUYUKI KANKE1, KATSUHARU SAITO1, HIROKAZU OKAYAMA1, SHOKI YAMADA2, SHOTARO NAKAJIMA1, EISEI ENDO1, KOJI KASE1, LEO YAMADA1, HIROSHI NAKANO1, TAKURO MATSUMOTO1, HIROYUKI HANAYAMA1, YOHEI WATANABE1, SUGURU HAYASE1, MOTONOBU SAITO1, ZENICHIRO SAZE1, KOSAKU MIMURA1, TOMOYUKI MOMMA1, SHINJI OKI1, YUKO HASHIMOTO2 and KOJI KONO1

Departments of 1Gastrointestinal Tract Surgery and 2Diagnostic Pathology, Fukushima Medical University, Fukushima 960-1295, Japan

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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 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

Correspondence to: Dr Yasuyuki Kanke, Department of Gastrointestinal Tract Surgery, Fukushima Medical University, 1 Hikarigaoka, Fukushima 960-1295, Japan
E-mail: kanke33@fmu.ac.jp

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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% H2O2 in methanol. For versican staining, 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 Envision+ 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 χ2 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 χ2 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.
Table I. Patient characteristics.

| Characteristic                          | Versican expression | Periostin expression | Lumican expression |
|-----------------------------------------|---------------------|----------------------|--------------------|
|                                         | High (n=50)         | Low (n=56)           | P-value            |
|                                         | Range (mean ± SD)   | Range (mean ± SD)    | Range (mean ± SD)  |
| Age, years                              | 42.81 (65.82±8.55)  | 37.79 (64.00±8.53)   | 0.8011             |
| ≤60, n                                  | 14                  | 18                   | 0.3755             |
| ≥61, n                                  | 36                  | 38                   | >0.999             |
| Sex, n                                  | 0.3755              | >0.999               | 0.4727             |
| Male                                    | 42                  | 51                   | 0.4727             |
| Female                                  | 8                   | 5                    | 0.4727             |
| Postoperative additional therapy, n     | 0.0436<sup>a</sup>  | 0.0529               | 0.0569             |
| No                                      | 36                  | 50                   | 49                 |
| Chemotherapy                            | 12                  | 6                    | 15                 |
| Radiotherapy                            | 2                   | 0                    | 2                  |
| Chemoradiotherapy                       | 0                   | 0                    | 0                  |
| Location, n                             | 0.2137              | 0.0409<sup>a</sup>   | 0.2884             |
| Upper                                   | 4                   | 11                   | 5                  |
| Middle                                  | 30                  | 31                   | 40                 |
| Lower                                   | 16                  | 14                   | 21                 |
| Invasion depth, n                       | <0.0001<sup>a</sup> | <0.0001<sup>a</sup>  | <0.0001<sup>a</sup>|
| pT1                                     | 25                  | 49                   | 34                 |
| pT2                                     | 10                  | 3                    | 13                 |
| pT3                                     | 14                  | 4                    | 18                 |
| pT4                                     | 1                   | 0                    | 1                  |
| Lymph node metastasis, n                | 0.0022<sup>a</sup>  | 0.0002<sup>a</sup>   | 0.0633             |
| Yes                                     | 27                  | 13                   | 34                 |
| No                                      | 23                  | 43                   | 32                 |
| Lymphatic invasion, n                   | 0.0002<sup>a</sup>  | 0.0049<sup>a</sup>   | 0.3773             |
| Yes                                     | 33                  | 16                   | 38                 |
| No                                      | 17                  | 40                   | 28                 |
| Venous invasion, n                      | <0.0001<sup>a</sup> | 0.0026<sup>a</sup>   | 0.0078<sup>a</sup> |
| Yes                                     | 40                  | 17                   | 46                 |
| No                                      | 10                  | 39                   | 20                 |
Results

Expression of versican, periostin and lumican in ESCC

as determined via immunohistochemistry. The clinico-
pathological characteristics of the 106 patients with ESCC
are summarized in Table SI. According to the expression of
versican, periostin and lumican, as assessed by immunohisto-
chemistry, the clinico-pathological 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 expres-
sion. 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 associa-
tion 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
clinico-pathological features, including a greater depth of inva-
sion, 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 expres-
sion 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 expres-
sion 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.

Table I. Continued.

| Characteristic | Versican expression | Periostin expression | Lumican expression |
|---------------|---------------------|----------------------|--------------------|
| Tumor differeniation, n |       |                      |                   |
|  | Well | Moderate | Poor | Unknown |  | Low (n=40) | High (n=66) | Low (n=40) | High (n=66) | Low (n=83) | High (n=23) | Low (n=83) | P‑value |
|  | 12  | 27    | 10   | 1       | 17 | 44    | 15    | 7     | 59  | 8     | 36     | 30     | 4      | 0.0091 |
|  | 26  | 54    | 17   | 12      | 36 | 44    | 8     | 7     | 1    | 3     | 11     | 1     | 1      | 0.0091 |
|  | 14  | 30    | 9    | 6       | 7  | 11    | 4     | 1     | 0    | 4     | 0      | 1     | 1      | 0.0091 |

TNM, Tumor-Node-Metastasis. Invasion depth (T-stage) was determined according to the TNM classification of Malignant Tumors 8th edition (16).

P<0.05.
(P=0.0931; Fig. S3), while stromal lumican was associated with RFS in stage I patients (Fig. S3).

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

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

|        | High (n=66) | Low (n=40) | P-value |
|--------|-------------|------------|---------|
| Versican |             |            |         |
| High (n=50) | 43          | 7          | <0.0001 |
| Low (n=56)  | 23          | 33         |         |

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

|        | High (n=23) | Low (n=83) | P-value |
|--------|-------------|------------|---------|
| Periostin |             |            |         |
| High (n=66) | 22          | 44         | <0.0001 |
| Low (n=40)  | 1           | 39         |         |

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

|        | High (n=50) | Low (n=56) | P-value |
|--------|-------------|------------|---------|
| Lumican |             |            |         |
| High (n=23) | 17          | 6          | 0.0076  |
| Low (n=83)  | 33          | 50         |         |

(P=0.0931; Fig. S3), while stromal lumican was associated with RFS in stage I patients (Fig. S3).
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 | 0.80| 0.30-2.15  | 0.6545  |
| Lymph node metastasis (yes vs. no) | 3.10| 1.44-6.71  | 0.0040  | 0.56| 0.19-1.64  | 0.2870  |
| Lymphatic invasion (yes vs. no) | 3.59| 1.57-8.22  | 0.0025  | 1.01| 0.37-2.79  | 0.9814  |
| Venous invasion (yes vs. no)  | 9.26| 2.78-30.82 | <0.0003 | 3.07| 0.78-12.09 | 0.1095  |
| TNM stage                     | 1.98| 1.42-2.75  | <0.0001 | 1.81| 1.03-3.16  | 0.0390  |
| Versican (high vs. low)       | 9.11| 3.14-26.44 | <0.0001 | 3.96| 1.16-13.46 | 0.0278  |
| Periostin (high vs. low)       | 7.46| 2.24-24.88 | 0.0011  | 1.91| 0.48-7.65  | 0.3582  |
| Lumican (high vs. low)         | 5.11| 2.35-11.0 | <0.0001 | 2.55| 1.06-6.17  | 0.0371  |

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

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 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, 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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Availability of data and materials

The datasets and materials used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YK and KKO conceived and designed the present study. SO and NY collected the data. YK, SO and NY confirm the authenticity of all the raw data. KS performed sample immunostaining. NY, KS, SY, YH, EE, KKa, LY, ZS, KM, TMa and YK performed the majority of the current study and analyzed the data. HO, HN, TMo, HH, YW, SH and MS participated in analyzing the data and drafting the initial manuscript. NY, YK, SN, KS and KKO revised the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The current study was approved by Fukushima Medical University Certified Review Board (Fukushima, Japan; approval no. 2329). Informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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