Cancer-associated fibroblasts (CAFs) promote the lymph node metastasis of esophageal squamous cell carcinoma

Hajime Kashima[1], Kazuhiro Noma[1], Toshiaki Ohara[1, 2], Takuya Kato[1], Yuki Katsura[1], Satoshi Komoto[1], Hiroaki Sato[1], Ryoichi Katsube[1], Takayuki Ninomiya[1], Hiroshi Tazawa[1, 3], Yasuhiro Shirakawa[1] and Toshiyoshi Fujiwara[1]

[1] Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

[2] Department of Pathology & Experimental Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

[3] Center for Innovative Clinical Medicine, Okayama University Hospital, Okayama, Japan

Address correspondence to: Dr. Kazuhiro Noma, Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan. Phone: +81-86-235-7255; Fax: +81-86-221-8775; E-mail: knoma@md.okayama-u.ac.jp.

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Abbreviations used in this article: AB, antibody; CAF, cancer-associated fibroblast; CM, conditioned medium; CI, confidential interval; DAB, 3,3'-diaminobenzidine; DAPI, 4,6-diamidino-2-phenylindole; DFS, disease-free survival; DMEM, Dulbecco's modified eagle medium; EC, esophageal cancer; ESCC, esophageal squamous cell carcinoma; FACS, fluorescence-activated cell sorting; FAP, fibroblast activation protein; FBS, fetal bovine serum; FEF3, fetal esophageal fibroblast; GFP, green fluorescent protein; HE, hematoxylin and eosin; HR, hazard ratio; IHC, immunohistochemistry; MMP, matrix metalloproteinase; OS, overall survival; RPMI, Roswell Park Memorial Institute; RT, room temperature; SEM, standard error of the mean; SMA, smooth muscle actin; SPSS: statistical package for the social sciences; TGF, transforming growth factor; TNM: tumor-node-metastasis; TME: tumor microenvironment; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium sodium.

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Novelty and Impact:

The presence of FAP^+ CAFs, identified by the FAP score, in clinical samples was strongly correlated with the lymph node metastasis of esophageal squamous cell carcinoma. Here, we clarified this relationship using esophageal cancer cell lines *in vitro* and orthotopic metastatic models. Targeted therapy against FAP^+ CAFs may reduce node metastasis and further tumor spreading, and improve the prognosis of patients with esophageal cancer in the future.

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Abstract

Lymph node metastasis is a pathognomonic feature of spreading tumors, and overcoming metastasis is a challenge in attaining more favorable clinical outcomes. Esophageal cancer is an aggressive tumor for which lymph node metastasis is a strong poor prognostic factor, and the tumor microenvironment (TME), and cancer-associated fibroblasts (CAFs) in particular, has been implicated in esophageal cancer progression. CAFs play a central role in the TME and have been reported to provide suitable conditions for the progression of esophageal cancer, similar to their role in other malignancies. However, little is known concerning the relevance of CAFs to the lymph node metastasis of esophageal cancer. Here, we used clinical samples of esophageal cancer to reveal that CAFs promote lymph node metastasis and subsequently verified the intercellular relationships *in vitro* and *in vivo* using an orthotopic metastatic mouse model. In the analysis of clinical samples, FAP^+ CAFs were strongly associated with lymph node metastasis rather than with other prognostic factors. Furthermore, CAFs affected the ability of esophageal cancer cells to acquire metastatic phenotypes *in vitro*; this finding was confirmed by data from an *in vivo* orthotopic metastatic mouse model showing that the number of lymph node metastases increased upon injection of co-cultured cancer cells and CAFs. In summary, we verified *in vitro* and *in vivo* that the accumulation of CAFs enhances the lymph node metastasis of ESCC. Our data suggest that CAF targeted therapy can reduce lymph node metastasis and improve the prognosis of patients with esophageal cancer in the future.

Introduction

The acquisition of metastatic properties is the one of the steps in the evolution of malignant cells, and consequently, the life of many patients is threatened by translocated neoplasms in vital organs, leading to cancer-related death. Lymph node metastasis is a pathognomonic feature of metastatic tumors, and overcoming metastasis is a challenge in our efforts to regulate tumor spread and attain more favorable clinical outcomes[1].

Esophageal carcinoma (EC) is the eight most common cancer worldwide; as one of the most aggressive malignant tumors, EC is the sixth most common cause of death from cancer[2]. Patients with lymph node metastasis have a worse prognosis than those without lymph node metastasis[3, 4]. In past decades, treatment of EC and molecular characteristics relating to its prognosis were mainly focused on targeting malignant tumor cells. However, similar to the recent innovation of immunotherapy for malignant tumors, targeting the tumor microenvironment (TME) has once again become a focus for cancer therapy[5-7]. Recent studies have revealed that EC progression is related to the TME, especially diffusible growth factors, cytokines, and various types of stromal cells[6, 8]. In the TME, cancer-associated fibroblasts (CAFs) are believed to play a central role in tumor progression.

CAFs secrete several signaling molecules, including inflammatory cytokines, growth factors and chemokines, to stimulate cancer cells in the TME, and they remodel the extracellular matrix (ECM) by secreting ECM components[8-11]. Through these properties, CAFs provide suitable conditions for tumor progression by promoting tumor growth, survival, angiogenesis, inflammation, drug resistance, invasion and metastasis[1, 5, 7]. Although activated CAFs in the TME may strongly contribute to the progression of EC, as they do in many other malignancies, CAFs have seldom been discussed in EC, and there

have been few reports concerning the correlation between CAFs and EC[12-14], especially on the influence of CAFs on lymph node metastasis or on CAFs as a strong prognostic factor[15]. A few reports thus far have provided mechanistic or signaling data related to metastasis[8, 16, 17], in which only the indirect contribution of CAFs to tumor metastatic properties has been clarified[9, 18, 19]. Of note, recent reports showed that heterotypic adhesion of CAFs and tumor cells drives cancer cell invasion[20] and that CAFs promote nodal metastases using xenograft model[21]. Thus, further investigations should be conducted using orthotopic model[22-25], which is more appropriate mimic of esophageal cancer, concerning direct cell-cell interactions involving CAFs that affect lymph node metastasis.

In this study, we reveal that the presence of CAFs in clinical samples of esophageal squamous cell carcinoma (ESCC) promotes lymph node metastasis, which is associated with prognosis. Furthermore, we verify the intercellular relationships *in vitro* and *in vivo* using an orthotopic metastatic model. Here, we propose that increased numbers of stromal CAFs may increase lymph node metastasis.

Materials and Methods

Patients and tissue samples

In total, 94 paraffin-embedded primary human ESCC specimens resected from 2008 to 2010 in Okayama University Hospital were included. This retrospective study was approved by the Institutional Review Board at Okayama University Hospital and conducted in accordance with the 1996 Declaration of Helsinki. All patients provided written informed consent according to institutional guidelines. No patient received preoperative chemotherapy or radiotherapy. Postoperative adjuvant treatment was administered to 35.1%

(33/94) of the patients: 9 patients received chemotherapy and radiotherapy, and 24 received chemotherapy alone. Clinical and pathological reports were reviewed for gender, age, tumor location, invasion depth (T), nodal status (N), histological grade, lymphatic invasion, venous invasion, and recurrence. The median follow-up time was 1582 days (range 14-2557 days), and 25 patients (26.6%) developed local or distant recurrence. Thirty-five patients (37.2%) died within the observation period. Overall survival (OS) was defined as the time from the date of the operation to the date of death or last follow-up. Disease-free survival (DFS) was measured from the date of the operation to the date of first evidence of relapse. For patients who had not relapsed or died, DFS was censored at the last date on which the absence of relapse was confirmed. TNM classification was applied according to guidelines from the 2009 Union for International Cancer Control staging manual (UICC 7th edition).

Antibodies

The following antibodies were used in this study: monoclonal anti-α smooth muscle actin (SMA) (Sigma-Aldrich, St Louis, MO) and monoclonal anti-fibroblast activation protein (FAP) (Novus, Littleton, CO) for immunohistochemistry (IHC); and polyclonal anti-αSMA (Abcam, Cambridge, UK), monoclonal anti-FAP (Abnova, Taipei, Taiwan), monoclonal anti-vimentin (Cell Signaling Technology, Danvers, MA, US), monoclonal anti-matrix metalloproteinase (MMP) 2 (Abcam, Cambridge, UK), and anti-β-actin (Sigma-Aldrich, St Louis, MO) for Western Blotting.

Immunohistochemical analysis

Sections on microslides were deparaffinized with xylene, hydrated using a diluted alcohol series, and immersed in H_2O_2 in methanol to quench endogenous peroxidase activity.

Sections were treated with citrate buffer solution. To reduce non-specific staining, each section was blocked with Serum-Free Protein Block (Dako, Agilent Technologies, Santa Clara, CA) for 15 min. The sections were then incubated at room temperature (RT) with anti-SMA antibody (1:1000 dilution) or anti-FAP antibody (1:200 dilution; Novus, Littleton, CO) diluted in Dako REAL Antibody Diluent (Dako, Agilent Technologies, Santa Clara, CA) for 30 or 60 min, respectively, followed by three washes with buffer. Sections were then incubated with Envision+ anti-mouse/rabbit antibody (Dako, Agilent Technologies, Santa Clara, CA) for 30 min at RT. The chromogen was liquid DAB+ (Dako, Agilent Technologies, Santa Clara, CA). Sections were counterstained with Meyer's hematoxylin.

Evaluation of FAP score

The overall percentage of stromal FAP staining in the primary tumor was assessed semi-quantitatively as a proportion score (0, no staining; 1, <10% staining; 2, <30%; 3, <60%; and 4, ≥60%), and the staining intensity was given an intensity score (0, none; 1, weak; 2, intermediate; and 3, strong). We used ImageJ (U.S. National Institutes of Health, Bethesda, MD) to evaluate the stromal FAP proportion score. The stromal area around cancerous tissue was reviewed under light microscopy at 40× magnification, and DAB staining (brown areas) was assessed with ImageJ to calculate the proportion score. The area of a microslide with the most staining was assessed to determine the intensity score. We graded the stromal intensity score visually. We defined the FAP score as the combination of the proportion and intensity scores; patients with a FAP score of 0 were placed in the FAP-negative group, those with a FAP score of 2-3 were in the FAP-low group, and those with a FAP score of 4-7 were in the FAP-high group. The analysis was evaluated by independent two doctors who were blinded to the clinicopathological characteristics and

outcomes of the patients.

Cell lines

We used human ESCC cells (TE1, TE4 and luciferase-expressing TE4 (TE4-luc)), a human esophageal adenocarcinoma cell line (OE19) and a human fetal esophageal fibroblast line (FEF3) in this study. All of these cancer cell lines were purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). FEF3 primary human esophageal fibroblasts were isolated from the human fetal esophagus as described previously. TE4-luc cells were transfected with luciferase using Lipofectamine® 3000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA). TE1, TE4 and OE19 cells were maintained in Roswell Park Memorial Institute (RPMI) medium 1640 (Sigma-Aldrich, St Louis, MO) supplemented with 10% (vol/vol) FBS (Gibco, Thermo Fisher Scientific, Waltham, MA), penicillin (100 units/mL) (Sigma-Aldrich, St Louis, MO), and streptomycin (100 units/mL) (Sigma-Aldrich, St Louis, MO) at 37 °C in a 5% (vol/vol) CO_2 incubator. The FEF3 cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St Louis, MO) supplemented with 10% (vol/vol) FBS, penicillin (100 units/mL), and streptomycin (100 units/mL) at 37 °C in a 5% (vol/vol) CO_2 incubator. All cell lines were authenticated by the JCRB Cell Bank (National Institute of Biomedical Innovation, Osaka, Japan) using short tandem repeat (STR) analysis.

Activation of esophageal cancer by conditioned medium (CM) from fibroblasts

EC cells were seeded in 10-cm dishes with RPMI containing 2% FBS, and after overnight attachment and growth, the cells were washed twice with PBS and then incubated in DMEM containing 2% FBS. CM was collected after 72 h (cells at 70%–80% confluence)

and centrifuged, and this CM was defined as CM/cancer (e.g. CM/TE4). FEF3 cells were incubated with this cancer CM for 48 h, and the resulting CM was collected and termed CM/CAF. A schema of this protocol was shown in Supporting Information Fig. S1. To activate EC cells, they were cultured with CM/CAF for 48 h. EC cells with normal media were defined as control.

Migration and invasion assays

For migration assays, 24-well cell culture inserts with a pore size of 8 μm (Falcon®, CORNING, Corning, NY) were used. In migration assays, transwell inserts were placed over a well containing CM/CAF or normal media. EC cells were then seeded into the upper compartment in serum-free fresh media and allowed to migrate for 24 h. Migratory cells pass through the membrane and attach to the bottom surface of the membrane. After cells remaining in the upper chamber were removed, migratory cells were stained with crystal violet, and images were captured in 5 different high-power fields at 200x magnification. The number of migratory cells was then counted visually. In invasion assays, we used transwell inserts coated with Matrigel® (CORNING, Corning, NY), and the subsequent procedures were the same as those for migration assays.

Western blotting

FEF3, cultured and stimulated with CM/Cancer for 72 h, were examined for the expression of CAF markers by Western blotting. Cells were washed with cold PBS, lysed in SDS buffer and centrifuged at 4 °C for 10 min. The supernatant was collected and subjected to Western blot analysis. Forty micrograms of protein from each lysate was fractionated by 10% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride

membranes (Millipore, Bedford, MA). After being blocked with Blocking One at RT for 1 h, membranes were incubated overnight at 4 °C with primary antibodies against αSMA, FAP, vimentin, MMP-2, or β-actin (1:1000 dilution) and then visualized using an ECL system (GE Healthcare UK Ltd.) according to the manufacturer's protocol. Membranes were then incubated at RT for 1 h with appropriate horseradish peroxidase-labelled secondary antibodies at a 1:2000 dilution. After TBS-T washes, the blot was incubated in ECL reagent (Amersham Bioscience, Freiburg, Germany). After being washed, membranes were visualized using a LAS-4000 mini imager (FUJIFILM). EC cells (0.5×10^6 cells) in Falcon Companion TC Plates (Catalog No. 353502, 6 wells) and FEF3 (0.2×10^6 cells) in Falcon Cell Culture Inserts (Catalog No. 353090) were seeded and cultured for 48 h respectively. And then, EC cells were collected. The expression of MMP2 was verified by WB, as described above.

Orthotopic lymph node metastatic model

Six-week-old female BALB/c nu/nu mice were purchased from CLEA (Tokyo, Japan) and housed under pathogen-free conditions. We established the esophageal orthotopic lymph node metastatic model by the following procedures. A 7- to 10-mm skin incision was made in the middle of the upper abdomen, and the stomach was pulled down so that the abdominal esophagus could be seen. A 1-ml syringe with a 30-gauge needle was used to inject cancer cells (TE4-luc and FEF3 cells) suspended in equal volume Matrigel (20 μ L) into the abdominal esophagus. TE4-luc cells (1.5×10^6) in 20 μ L were injected into mice in the control group, whereas TE4-luc cells (1.5×10^6) activated by CM/CAF and FEF3 cells (0.5×10^6) activated by TE4-luc CM in 20 μ L were co-injected into mice in the CAF group.

At 6 weeks after tumor inoculation, all the mice were sacrificed and observed.

Evaluation of luciferase activity in the orthotopic lymph node metastatic mouse model

To assess the effect of CAFs on tumor metastasis, we used with an IVIS Lumina imaging system (Xenogen IVIS Lumina II; Caliper LifeSciences, Hopkinton, MA), and image analysis and bioluminescent quantification were performed by using Living Image software. Every week after inoculation, the luminescence intensity of metastatic nodules in the mice was measured. To analyze the luminescence intensity, the mice received an intraperitoneal injection of 200 mg of *In Vivo*-Grade VivoGlo Luciferin (Promega, Madison, WI) and were then imaged under isoflurane anesthesia after 10 min. We evaluated metastases in the abdominal cavity and counted the number of metastatic nodules in lymph nodes in the abdominal cavity.

Statistics

The correlation of FAP expression with clinicopathological features was examined using Pearson's chi-square test or t-test as appropriate, and Spearman's rank correlation coefficients were calculated. Cumulative survival (OS and DFS) was determined using the Kaplan–Meier method and compared using the log-rank test. The Cox proportional hazards model was used for univariate and multivariate analyses. The hazard ratio (HR) and 95% confidence interval (CI) for each clinical or pathological factor were calculated using Cox proportional hazards regression in univariate and multivariate analyses. The significance of the relationship of each factor with lymph node metastasis was evaluated as the OR by binary univariate and multivariate logistic regression analyses. Factors that were statistically significant in the univariate analysis were included as co-variables in the multivariate

analysis. Statistical analysis *in vitro* and *vivo* was performed using Fisher's exact test or t-test as appropriate. $P<0.05$ indicated statistical significance. All statistical analyses were performed using IBM SPSS Statistics (IBM, Chicago, IL, USA).

Results

Presence of FAP-positive CAFs in tumor stroma correlates with clinical outcomes of patients with esophageal squamous cell carcinoma.

First, we examined FAP expression in clinical samples of EC. FAP was specifically expressed in tumor stroma, whereas SMA-positive cells were also observed in the normal smooth muscle layer and microvessel wall of the human esophagus; thus, it was difficult to distinguish muscle cells from activated fibroblasts in the tumor stroma, especially in advanced cases (Fig. 1a). We then evaluated FAP expression in the tumor stroma using the proportion (Fig. 1b) and intensity (Fig. 1c) scores based on the Allred score[26]. All cases were divided into three groups according to FAP score: greater than or equal to four, high; one to three, low; and zero, negative. As shown in the FAP expression graph (Fig. 1d), there were twenty-one cases with a FAP score of 0, ten with a score of 2, thirteen with a score of 3, seventeen with a score of 4, eleven with a score of 5, seventeen with a score of 6 and five with a score of 7. We analyzed the prognostic factors, including the FAP score, for EC (Supporting Information Table S1). Even though the FAP score was not an independent prognostic factor in the multivariate analysis, TNM factors and FAP score showed statistical significance as prognostic factors for ESCC in the univariate analysis, as we expected. In our study, only T factor was a strong prognostic factor in the multivariate analysis. However, the FAP score was significantly correlated with several clinical features,

such as tumor depth (p<0.01), lymph node metastasis (p<0.01), venous invasion (p<0.01) and lymphatic invasion (p<0.01) (Table 1). These results may indicate that FAP-positive cells are related to these strong prognostic factors, resulting in worse prognosis. In fact, FAP expression was significantly correlated with shorter OS and DFS. In particular, the FAP-high group had significantly worse 5-year DFS (58.6%; Fig. 1e) and OS (49.7%; Fig. 1f) than the FAP-low and FAP-negative groups (DFS: 80.4% and 100%, p=0.017; OS: 72.4% and 77.5%, p=0.002).

FAP-positive CAFs are associated with lymph node metastasis of esophageal squamous cell carcinoma.

Lymph node metastasis is strong prognostic factor for EC. The 5-year OS (Fig. 2a) and DFS (Fig. 2b) of the node-positive group were significantly worse than those of the node-negative group (OS: HR 3.86, 95% CI: 1.94-12.7, p=0.001; DFS: HR 4.97, 95% CI: 1.89-7.89, p<0.001). The correlations were verified to investigate whether stromal FAP-positive CAFs are implicated in lymph node metastasis. First, the proportion of lymph node-positive cases was evaluated according to the FAP score, and the results showed a gradual increase in the number of lymph node-positive cases as the FAP score increased: FAP score 0, one lymph node-positive case of 21 total cases (4.7%); score 2, one of 10 (10.0%); score 3, five of 13 (38.5%); score 4, 12 of 17 (70.5%); score 5, seven of 11 (63.6%); score 6, eight of 17 (47.1%); and score 7, four of five (80.0%). When the cases were divided into two groups based on lymph node positive or negative status, the average FAP score was 2.4 for the negative cases and 4.6 for the positive cases; this difference was significant (p<0.0001; Fig. 2d). Furthermore, in the correlation analysis, FAP score showed a significant correlation with the number of nodal metastases (r=0.448, p<0.001; Fig. 2e).

To evaluate the risk factors for lymph node metastasis, we performed statistical analysis using a (binary) logistic regression model. FAP score was an independent risk factor for nodal metastasis (odds ratio: 3.84, 95% CI: 1.50-9.86, p=0.005) in the multivariate analysis (Table 2). These results indicated that the presence of FAP-positive CAFs may strongly affect lymph node metastasis in ESCC, resulting in the correlation with worse patient prognosis.

Activated fibroblasts enhance the migration and invasion of esophageal cancer cells *in vitro*.

To evaluate the impact of CAFs on EC cell lines, we investigated morphological changes in cancer cells and their migratory and invasive abilities *in vitro*. First, we determined whether our CAF model activated by EC cell lines expressed SMA, FAP and vimentin, which are generally used as specific markers of CAFs. FEF3 cells activated by EC cells over-expressed SMA, FAP and vimentin compared to control FEF3 cells (Fig. 3a). Bright-field microscopy demonstrated that cancer cells (TE1, TE4 and OE19) stimulated by activated CAFs lost cell-cell adhesions and adopted a spindle cell-shaped morphology (Fig. 3b), whereas there was little change in CM/FEF3 (Supporting information Fig. S2a). The migration ability of cancer cells was significantly increased upon culture with CM/CAF (Fig. 3c), as shown by an increased number of migratory cells in transwell chamber assays. The invasive ability of EC cells in Matrigel-coated transwell chambers was increased (Fig. 3d), similar to the findings for the migration assay (p<0.05.). Although CM/CAF had the strongest impacts on EC cells, normal fibroblasts (CM/FEF3) also had a certain effect compared to control (Supporting information Fig. S2b and c). Using Western blot analysis, we observed markedly increased expression of MMP2, which plays an important role in

tumor invasion and metastasis, in response to CAF activation (Fig. 3e).

CAFs promote the metastasis of esophageal carcinoma *in vivo*.

We next explored the effect of CAFs on lymph node metastasis of EC *in vivo*. Luciferase-labeled TE4 cells alone or mixed with activated FEF3 cells were orthotopically inoculated into nude mice, which have been established as an orthotopic tumor model, and we compared the control group (TE4 cells alone) to the CAF group (TE4 cells co-cultured with FEF3 cells). Esophageal tumors formed and could be detected two weeks after cell inoculation in all mice. There was no significant difference in mean body weight between the two groups at the study end-point. The mice were subjected to an *in vivo* luciferase assay to monitor metastasis every week after cell inoculation. Figure 4a shows a representative IVIS-generated picture of each mouse. Mice in the control group had luciferase luminescence in only the upper abdominal area, indicative of the inoculated esophageal tumor. In contrast, mice in the CAF group had luminescence in other parts of the body, indicating that these mice had metastatic tumors in addition to the primary orthotopic tumor. The total luminescence intensity in the CAF group was relatively higher than that in the control group beginning at three weeks after cancer cell inoculation (p=0.30 at six weeks) (Fig. 4b). To accurately evaluate the metastatic burden, we performed a laparotomy on sacrificed mice to make intraperitoneal observations and calculated the luminescence intensity of the metastatic areas by subtracting the luminescence intensity of the primary esophageal area from the total intensity. The CAF group had greater luminescence intensity in metastatic sites than the control group (p=0.06; Fig. 4c). To confirm the effect of CAFs on co-cultured tumors, we observed sections of primary esophageal tumors by microscopy and found that tumors from the CAF group had more

SMA-positive stromal cells than did those from the control group (Fig. 4d), confirmed by quantification of SMA expression[27] (Supporting information Fig. S3). Furthermore, we analyzed the sites and number of metastases. The CAF group had multiple metastases and metastatic sites, as shown in Fig. 4e. In the control group, three of the five mice had metastasis: one mouse of three had lymph node metastasis and peritoneal dissemination and another had only liver metastasis, and the other had only lymph node metastasis. In the CAF group, all five mice had metastasis, including lymph node metastasis in all five, liver metastasis in two, and peritoneal dissemination in three. Thus, the CAF group had a remarkably high incidence of lymph node metastasis ($p<0.01$; Fig. 4e, f). Next, to obtain more detailed information on lymph node metastasis, we examined the number of metastatic tumor nodules in the lymph nodes using IVIS and hematoxylin and eosin (HE) staining (Fig. 4g). The number of metastatic nodules in the lymph nodes was higher in the CAF group than in the control group (Fig. 4h), consistent with the clinical analysis. Taken together, these data suggest that CAFs markedly enhance tumor lymph node metastasis, which results in the poor prognosis of patients with EC.

Discussion

Our study demonstrates that CAFs, identified herein as FAP-positive stromal cells, correlate with clinical outcome in ESCC, especially with lymph node metastasis and prognosis. These results were further confirmed in our CAF model using fetal esophageal fibroblasts activated by EC cells, which showed the effect of CAFs on the metastatic properties of cancer cells *in vitro* and their ability to promote multiple metastases in the lymph nodes, liver and peritoneum in an *in vivo* orthotopic mouse model.

Heterogeneity of CAFs in the TME has been commonly reviewed since CAFs have multiple phenotypes, as evidenced by the specific expression of various markers, and heterogenic functions[20, 28]. In general, SMA-positive fibroblasts are called myofibroblasts, which are believed to be equal to CAFs, even though the myofibroblasts in the TME are considered a subtype of CAFs[29, 30]. FAP is a type II integral membrane serine protease of the prolyl oligopeptidase family[31]. FAP is not typically expressed in normal tissue but is expressed in activated fibroblasts, such as CAFs and those involved in wound healing and granulation, and in human bone marrow-derived mesenchymal stem cells[32]. In this study, we focused on FAP because there are many other SMA-expressing cells in the TME, such as vascular smooth muscle cells and smooth muscle cells of the muscularis mucosa layer and lamina propria in the gastrointestinal wall. In order to evaluate the presence of CAFs specifically in the TME, we selected FAP as a marker of CAFs[31, 33, 34] because several reports have shown that FAP^+ CAFs enhance tumor immunosuppression[35-37] and tumor growth by remodeling tumor-promoting desmoplasia[38]. Furthermore, we believe that in the TME, CAFs should be fibroblasts and distinguished from pericytes, smooth muscle cells, and endothelial cells, even though some reports have shown that cancer cells also express FAP, which can be a prognostic marker[39, 40]. To resolve this issue, we identified activated fibroblasts in the TME using FAP, since cancer cells can be differentiated from stromal cells using HE staining, even without double-immunofluorescent staining. Considering stomal heterogeneity, we therefore conclude that FAP^+ stomal cells should be evaluated regarding clinical outcomes in the future, although there are various subpopulations of CAFs.

Regarding the method of evaluating FAP expression status in this study, we adopted a FAP scoring system that combines two previously reported methods based on the

intensity and proportion/quantification theories[12, 41-43]. Several famous studies have used the semi-quantification method, with grades from 0 (-) to 3 (++)[41, 44-46], but the protease FAP has functional enzymatic activity during tumor progression. In this study, we therefore added the intensity score to the proportion/quantification score, for a possible total score of 0 to 7, in order to evaluate FAP^+ CAFs from a more multidirectional view. As expected, our scoring system revealed three groups with different OS and DFS curves, indicating that the FAP score is a convincing prognostic marker and that FAP intensity and quantification may be associated with patient survival or strongly correlate with tumor progression.

Our clinical analysis revealed that several pathological features, such as T, N, lymphovascular invasion and FAP score, are significant prognostic factors in ESCC, in line with previous findings[31, 40-42, 46, 47]. Among these features, only T factor was strongly associated with prognosis in the multivariate analysis; FAP score showed no such significant association. This finding makes sense because CAFs would not be needed for the initial acquisition of malignant phenotypes, as genetic alterations are; rather, they might be indirectly related to patient prognosis as they proliferate or accumulate simultaneously with cancer progression, suggesting that the feature of CAF accumulation ultimately depends on tumorigenesis. However, from the point of view of CAFs shown in Table 1, prognostic factors showed a significant correlation with CAF score, indicating that CAFs strongly influence certain prognostic factors. In particular, LN metastasis, which is the main focus of this study, was significantly correlated with FAP score in the multivariate analysis. Furthermore, our *in vitro* data confirmed that cancer cells acquire metastatic behavior upon activation by CAFs, and these findings were supported by the results of the *in vivo* orthotopic mouse model.

Our orthotopic tumor model used in this study revealed that CAFs are strongly

associated with tumor metastasis in the gastrointestinal area. Previously reported metastatic mouse models have been mostly subcutaneous tumor models[15, 21]. Furthermore, there are a few studies, which have reported orthotopic metastatic mouse models of esophageal cancer [22-25], however, of our knowledge, other orthotopic metastatic mouse models of co-cultured EC with CAFs have not been reported[48-50]. We have established and reported this esophageal orthotopic xenograft model, in which tumor volume and metastatic activity were evaluated by luciferase emission[51]. In this study, we successfully transfected TE4 cells with luciferase and applied TE4-luc cells to evaluate metastatic activity. Although our model revealed that co-cultured cells metastasize to the liver, lymph nodes and peritoneum more than cancer cells alone, all co-cultured tumors intriguingly showed locoregional lymph node metastasis and a significant increase in the number of lymph node metastases. These data are consistent with the results of our clinicopathological analysis, in which a correlation was observed between CAFs and the number of metastatic lymph nodes. Thus, it is possible that a greater presence of CAFs in EC leads to increased lymph node metastasis. Overall, our study provides data consistent with our hypothesis, from the clinical aspects to the *in vitro* and *in vivo* studies, and therefore suggests that CAFs accelerate tumor metastasis, especially to the lymph nodes.

Our results presented herein indicate the possibility of a correlation between FAP^+ CAFs and lymph node metastasis in ESCC. We have shown this phenomenon in clinical samples and *in vitro* and *in vivo* studies, but we do not reveal the mechanism by which CAF accumulation promotes lymph node metastasis. In our *in vitro* studies, we have demonstrated only MMP2 over-expression as confirmation of the acquisition of metastatic features, although many other signaling pathways would be involved. To evaluate the mechanism of interaction of CAFs and tumor cells, recent advances of imaging technology

would be useful as their heterogeneous host-tumor interactions[52-54]. Therefore, further investigations should be performed including using patients-derived xenografts[55]. However, we suggest that cellular targeted therapy for CAFs should be investigated in the future; various types of targeted therapies for inhibiting the interaction between tumor cells and CAFs have already been investigated, but most, including FAP targeted therapies, have not resulted in positive clinical outcomes[56, 57]. Our experimental procedures *in vivo* herein could be useful for clarifying the effect of CAFs at the cellular level and for evaluating the strategy of targeting CAFs. We speculate that CAF could be required to promote metastasis and survive in the metastatic deposits, based on the accumulation of FAP^+ CAFs in metastatic sites (Supporting Information Fig. S4 and S5) and that CAF targeted therapy will be effective against further spreading tumor, resulted in improving the prognosis of EC patients in the future. As a glimmer of hope, a recent study reported that FAP targeted therapy with chimeric antigen receptor T cells could control tumor progression *in vivo*. However, CAF targeted therapy may induce adverse events such as anemia and cachexia[37, 38], and the depletion of specific subpopulations may have either therapeutic or detrimental effects[38], thus necessitating further investigations. Furthermore, it is still great issue of whether accumulation of normal fibroblasts or the presence of CAFs contributes to tumor progression, since in fact there were not strong differences between co-culturing with normal fibroblasts and pre-treated fibroblasts (CAFs) for malignant phenotype of tumor (Supporting information Fig. S2b, c), and tumor growth (data not shown), suggested that even normal fibroblasts have strong effects to tumor cells. Accumulation of normal fibroblasts or “fibroblasts rich” condition rather than the presence of CAFs could promote tumor metastasis, since they would be activated to CAFs immediately after co-presence of tumor cells. These issues should be explored as the further projects in the future.

In conclusion, our data suggest that the accumulation of FAP-positive CAFs enhances the lymph node metastasis of ESCC. CAF targeted therapy can reduce spreading tumor and improve the prognosis of EC patients in the future.

Declarations

Ethics approval and consent to participate

The use of pathologic samples was approved and reviewed by the Ethics Review Board of Okayama University, Okayama, Japan (No. 1603-023). Details have been removed from these case descriptions to ensure anonymity. The animal experimental protocol was approved and reviewed by the Ethics Review Committee for Animal Experimentation of Okayama University, Okayama, Japan.

Consent for publication

All authors read and approved the manuscript. All contributing authors approved the submission of this version of the manuscript and asserted that the document represents valid work. No contributing authors have any disclosures to make.

Availability of data and materials

The datasets generated and/or analyzed during this study are included in this published article and are otherwise available from the corresponding author upon reasonable request.

Competing Interests

The authors declare no conflicts of interest.

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Author contributions

Conception and design: K. Noma, T. Ohara, H. Tazawa, Y. Shirakawa and T. Fujiwara.

Development of methodology: H. Kashima and K. Noma.

Data acquisition (provided animals, acquired and managed patients, provided facilities, etc.): H. Kashima, Y. Katsura, T. Kato, H. Sato and S. Komoto.

Data analysis and interpretation (e.g., statistical analysis, biostatistics, computational analysis): H. Kashima, T. Kato and K. Noma.

Writing, review, and/or revision of the manuscript: H. Kashima, K. Noma and T. Fujiwara.

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Kashima, K. Noma, H. Tazawa and T. Fujiwara.

Study supervision: K. Noma, T. Ninomiya, H. Tazawa, Y. Shirakawa and T. Fujiwara.

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Figure legends

Figure 1. Presence of FAP-positive CAFs in tumor stroma correlates with clinical outcomes of patients with esophageal squamous cell carcinoma (ESCC). (a) Comparison of FAP staining and SMA staining of esophageal cancer tissue. Normal human esophageal muscle and CAFs are stained by IHC for SMA, highlighting the difficulty in distinguishing activated fibroblasts from muscle layers in stroma. Scale bar, 200 μm. (b) Schema of FAP staining of primary tumors assessed by semi-quantitative methods (0, no staining; 1, <10% staining; 2, <30%; 3, <60%; and 4, ≥60%). (c) Representative microscopic images of FAP staining of esophageal stroma graded by intensity. Scale bar, 200 μm. (d) Distribution of patients according to FAP score. (e, f) FAP status is defined as “high” for a score greater than or equal to four, “low” for a score of two or three, and “negative” for a score of zero. Data were analyzed by a Cox regression hazard model, 95% confidence intervals and log-rank test. (e) Overall survival of esophageal cancer patients according to FAP status. (f) Disease-free survival of esophageal cancer patients according to FAP status.

Figure 2. FAP-positive CAFs are associated with lymph node metastasis in ESCC. (a, b) The lymph node metastasis-positive group is defined as "N positive", and the negative group is defined as "N negative". Data were analyzed by a Cox regression hazard model, 95% confidence intervals and log-rank test. (a) Disease-free survival of esophageal cancer patients according to lymph node metastasis status. (b) Overall survival of esophageal cancer patients according to lymph node metastasis status. (c) Proportion of patients according to FAP score. (d) Comparisons based on negative or positive lymph node metastasis status are shown by box-and-whisker plots. The bottom and top of the box represent the first and third quartiles, and the band inside the box indicates the median FAP score. The ends of the whiskers show the local minimum and local maximum (except for outliers). Cross marks show the mean FAP score. Lymph node metastasis-positive patients have a higher FAP score than lymph node negative-patients (p<0.0001; t-test) (e) Scatter plot shows the correlation between FAP score and the number of LN metastases. FAP score is on the X axis, and the number of metastatic lymph nodes in cancer patients is on the Y axis. FAP score positively correlated with the number of lymph node metastases (r=0.448, p<0.001; Spearman's rank correlation coefficient).

Figure 3. Activated fibroblasts enhance the migration and invasion of esophageal cancer cells *in vitro*. (a) Western blot analysis shows the expression of markers of CAFs stimulated by esophageal cancer cell lines. The expression of SMA, FAP and vimentin in FEF3 cells was increased upon stimulation by condition medium of cancer cells (TE1, TE4 and OE19). (b) Esophageal cancer cells (TE1, TE4 and OE19) acquired a mesenchymal phenotype after stimulation with CM/CAF, compared to control (regular medium). (upper, 100x magnification; lower, 400x magnification) (c, d) Migration and invasion ability of

esophageal cancer cells. The migration (c) and invasion (d) of esophageal cancer cells cultured with conditioned medium from CAFs (CM/CAF) were assessed by transwell assays. Both migratory and invasive abilities were increased by CM/CAF in transwell chamber assays (*p<0.05, t-test; 200x magnification), compared to control (regular medium). (e) Western blot analysis shows MMP2 over-expression in esophageal cancer cells treated with CM/CAF.

Figure 4. CAFs promote the metastasis of esophageal carcinoma *in vivo*. (a) Representative IVIS-generated pictures of mice. IVIS images were obtained once a week for 5 weeks after the orthotopic inoculation of TE4-Luc cells (control group) or of TE4-Luc cells and FEF3 cells (CAF group). Mice in the control group had luciferase luminescence in only the upper abdominal area, indicative of the orthotopic esophageal tumor. In contrast, mice in the CAF group had other luminescent areas in addition to those of the esophageal tumor, indicating metastatic sites. (b) The luminescence intensity of the whole tumor in each mouse was quantified every week. Bars in the graph indicate the standard error of mean (6 weeks after inoculation, p=0.30, t-test). (c) Comparison of luminescence intensity of metastatic tumors. Luminescence intensity of metastatic tumors was calculated by subtracting the luminescence of the main tumor from that of all tumors at 6 weeks after inoculation (p=0.06, t-test). (d) Microscopic images of the primary tumors show that tumors from the CAF group had more stromal cells than those from the control group by HE staining. IHC staining for SMA shows that the CAF group had more SMA-positive cells than the control group. HE, 200x magnification; IHC for SMA, 200x magnification; scale bar, 100 μm. (e) IVIS analysis of mice after laparotomy shows luciferase signals, indicating metastatic nodules in the lymph node (yellow arrows), liver (red arrows) and peritoneum (blue arrows) in addition

to the orthotopic esophageal tumors. (f) Incidence of orthotopic tumor metastasis to different organs. There was a significantly higher incidence of lymph node metastasis in the CAF group ($p<0.01$, Fisher's exact test). (g) IVIS shows that tumors emitted luminescence in the resected stomach and countable metastases emitted luminescence on the liver and lymph nodes. (h) Comparison of the number of lymph node metastases. There was a greater number of lymph node metastases in the CAF group than in the control group ($*p<0.01$, t-test). (c, h) The bottom and top of the box represent the first and third quartiles, and the band inside the box indicates the median value. The ends of the whiskers show the local minimum and local maximum (except for outliers). Cross marks show the mean value.