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Phosphorylation of Fascin Decreases the Risk of Poor Survival in Patients With Esophageal Squamous Cell Carcinoma

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SUMMARY Phosphorylation of fascin at serine 39 (phospho-S39-fascin) could inhibit its actin-binding and actin-bundling activities and decrease filopodia formation. However, the relationship between phospho-S39-fascin expression and clinicopathological parameters in tumors is still unknown. Here, Western blot analysis and IHC applied to tissue microarray technology were performed to examine the expression status of non-phosphorylated fascin (fascin) and phospho-S39-fascin and their impacts on the prognosis of patients with esophageal squamous cell carcinoma (ESCC). Fascin and phospho-S39-fascin expressions were tested by cytoplasmic staining. Among the 254 patients, 90 cases showed high expression of fascin and 87 cases showed high expression of phospho-S39-fascin. Survival analysis showed that high expression of fascin was significantly associated with a poor prognosis of the patients with ESCC ($p=0.004$). In contrast, high expression of phospho-S39-fascin correlated significantly with an improved outcome of patients ($p=0.020$). Multivariate analysis showed that both fascin and phospho-S39-fascin were independent prognostic factors. In a combined analysis, the patients with high expression of fascin and low expression of phospho-S39-fascin tumors had a shorter overall survival than those with high expression of both fascin and phospho-S39-fascin tumors (5-year overall survival rate: 28.7% vs 48.3%, $p=0.068$). Our results suggest that high expression of fascin correlates with poor outcome and that high expression of phospho-S39-fascin decreases the risk of poor prognosis in ESCC. This manuscript contains online supplemental material at <http://www.jhc.org>. Please visit this article online to view these materials. (J Histochem Cytochem 58:979–988, 2010)

KEY WORDS

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ESOPHAGEAL CARCINOMA is the sixth leading cause of cancer-related deaths worldwide and the fourth leading cause of cancer deaths in China (Enzinger and Mayer 2003; Parkin et al. 2005). More than 90% of cancers are either esophageal squamous cell carcinomas (ESCCs) or adenocarcinomas (Daly et al. 2000). In China, squamous cell carcinoma is the most common histological type of cancer. Because of the difficulty of early detection and metastatic recurrence in the advanced stage, patients

with ESCC have a high mortality. Unfortunately, efficient prognostic markers remain rare.

Fascin, a structurally unique and evolutionarily actin-crosslinking protein, could induce the membrane protrusions and increase cell motility of epithelial cells (Yamashiro et al. 1998). It was named fascin because of its ability to form tight and stable bundles with F-actin (Kureishy et al. 2002). Fascin is absent or shows very low expression in normal epithelium but is upregulated

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in transformed cells and in many kinds of human neoplasms, such as esophageal (Hashimoto et al. 2005; Xue et al. 2006), gastric (Hashimoto et al. 2004), colonic (Hashimoto et al. 2006), pancreatic (Yamaguchi et al. 2007), lung (Pelosi et al. 2003), breast (Rodríguez-Pinilla et al. 2006), ovarian (Daponte et al. 2008), prostate (Darnel et al. 2009), and thyroid (Chen et al. 2008) tumors, and high expression of fascin is associated with poor survival of patients.

In our previous work, we found that fascin may be correlated with the malignant transformation and development of normal esophagus to carcinoma. Fascin was upregulated in the progression of immortalized human esophageal epithelial cells to malignantly transformed esophageal cells (Shen et al. 2000; Rong et al. 2004). Immunohistochemical analysis also showed that upregulation of fascin occurred early in the progression from normal epithelium to carcinoma, and this might be a potential biomarker for early-stage ESCC (Zhang et al. 2006). Furthermore, the experiments *in vivo* and *in vitro* showed that silenced fascin decreased the formation of dynamic cell protrusions and resulted in a suppression of cell proliferation and invasiveness by affecting the expressions of CYR61 and CTGF via transforming growth factor- β pathway (Xie et al. 2005, 2010). These results suggested that fascin may play crucial roles in regulating neoplasm progression of ESCC by participating in the formation of cell protrusions and proliferation.

Phosphorylation of fascin induced the disappearance of stress fibers and membrane ruffles in human neuroblastoma cells, which caused cells to be rounded. Phosphorylation of human fascin purified from HeLa cells greatly reduced its actin-binding and actin-bundling activities (Yamakita et al. 1996). The major phosphorylation site was located at serine 39 within the *N*-terminal actin-binding domain of human fascin. Phosphorylation of fascin at serine 39 (phospho-S39-fascin) regulated its actin-binding ability and localization in cells (Ono et al. 1997; Adams et al. 1999). Recent studies using expression of phosphomimetic fascin mutants proposed that phosphorylation of fascin reduced filopodia frequency and acted as a molecular switch for filopodia formation. Expression of the active phospho-ablated fascin mutant S39A increased the number and length of filopodia in cells, whereas the inactive phospho-mimicking fascin mutant S39D/S39E markedly reduced the formation of filopodia and caused loose bundles (Adams et al. 1999; Vignjevic et al. 2006; Aratyn et al. 2007). The results of these studies indicated that phospho-S39-fascin had an opposite effect on the formation of filopodia compared with non-phosphorylated fascin. However, to date, the expression of phospho-S39-fascin and its role in the progression of tumors are still unclear.

In this study, we evaluated non-phosphorylated fascin (fascin) and phospho-S39-fascin expressions

and their impacts singly and in combination on the outcome of patients with ESCC.

Materials and Methods

Generation of Phospho-S39-fascin-specific Antibodies

The rabbit anti-human phospho-S39-fascin was raised against a synthetic peptide corresponding to aa 33–42 of serine 39-phosphorylated fascin [VNASAS(p-S) LKK, human]. Peptide synthesis was performed from C-terminus to N-terminus by using Fmoc chemistry and a solid support resin. Synthesized crude peptides were purified and examined by HPLC and C18 reversed-phase chromatography. Purified peptides were conjugated to the carrier protein keyhole limpet hemocyanin (KLH). Peptide immunogens conjugated to KLH were used to generate immune responses in rabbits (pathogen-free, barrier-raised New Zealand White Rabbits). Immunizations and serum collections were performed using an 80-day immunization protocol; after an adsorption by corresponding non-phosphorylated peptide (VNASASSLKK, human), the sera were purified using proprietary peptide affinity chromatography techniques.

Cell Lines and Cell Culture

Human esophageal squamous carcinoma cell lines KYSE150, KYSE180, KYSE510, EC109, EC171, EC8712, EC9706, and SHEEC were cultured in 199 medium (Invitrogen; Carlsbad, CA) or DMEM (Invitrogen) containing 10% fetal calf serum.

Sample Collection and Tissue Microarray Construction

For the retrospective study, immunohistochemical staining was performed on 254 paraffin-embedded specimens surgically resected at Shantou Central Hospital from 1987 to 1997. Among these patients, 64 cases were female and 190 cases were male. The median age was 55 years with a range of 32–73 years. The patients were followed up for a maximum period of 131.3 months and a median of 25.15 months. Information on gender, age, stage of disease, and histopathological factors was obtained from the medical records. Patients' data are summarized in Table 1. For Western blot analysis, tissue specimens and paired adjacent normal epithelial tissues were obtained from 18 patients with primary ESCC who underwent surgery in the same hospital. The patients who suffered from severe postoperative complications and those who died of other tumors or other causes were excluded. The study was approved by the local ethics committee.

For tissue microarray (TMA) construction, representative regions of each tissue were selected from paraffin blocks according to hematoxylin and eosin-stained sections without prior knowledge of fascin or phospho-S39-fascin immunostaining. Two tissue cores were obtained from each specimen measuring 1.8 mm in diameter

Table 1 Patient characteristics

Characteristics	No.	5-Year survival rate (%)	p Value of survival
Total no. with assessable stains	254	40.1	–
Median age (range; years)	55 (32–73)	–	–
Median follow-up (range; months)	25.15 (1.7–131.3)	–	–
Age			
<55 years	122 (48.0%)	43.9	0.399
≥55 years	132 (52.0%)	36.5	
Gender			
Female	64 (25.2%)	46.8	0.238
Male	190 (74.8%)	37.8	
Location			
Upper	11 (4.3%)	70.7	0.284
Middle	159 (62.6%)	37.2	
Lower	84 (33.1%)	42.3	
Tumor size			
<5 cm	139 (54.7%)	44.5	0.010*
≥5 cm	115 (45.3%)	34.9	
Histological grade			
Well differentiated	54 (21.3%)	61.9	0.015*
Moderate differentiation	162 (63.8%)	36.7	
Poor differentiation	38 (14.9%)	24.8	
Primary tumor			
T1 or T2	36 (14.2%)	48.9	0.082
T3 or T4	218 (85.8%)	38.6	
Regional lymph nodes			
N0	137 (53.9%)	56.5	0.000*
N1	117 (46.1%)	20.2	
Distant metastasis			
M0	241 (94.9%)	41.7	0.016*
M1	13 (5.1%)	15.4	
pTNM stage			
I or II	135 (53.2%)	54.4	0.000*
III or IV	119 (46.8%)	23.7	
Therapy			
Only surgery	154 (60.6%)	43.8	0.500
Surgery + chemo	50 (19.7%)	37.3	
Surgery + radio	41 (16.1%)	32.0	
Surgery + chemo + radio	9 (3.5%)	33.3	

*p Values <0.05 were considered significant.
Chemo, chemotherapy; radio, radiotherapy.

and ranging in length from 1.0 to 3.0 mm, depending on the depth of tissue in the donor block. Each core was precisely arrayed into a new paraffin block. These microarrays were serially sectioned (4 μ m) and stained with hematoxylin and eosin to verify tissue sampling and completeness. Unstained sections were baked overnight at 56°C in preparation for IHC.

Immunohistochemical Staining

Tissue microarray sections were cut at 4 μ m from the TMA blocks, dewaxed in xylene, rehydrated in alcohol,

and incubated in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Antigen retrieval was performed by microwave oven heating (10 min) in 0.01 M sodium citrate buffer (pH 6.0). Sections were incubated with 10% normal goat serum in PBS for 15 min at room temperature to block nonspecific binding. Then sections were incubated overnight at 4°C with primary antibodies for mouse anti-human fascin-1 (monoclonal, 55K-2, 1:100 dilution; Dako, Carpinteria, CA) and rabbit anti-human phospho-S39-fascin (polyclonal, 1:100 dilution; Beijing Biosynthesis Biotechnology, Beijing, China). After rinsing with PBS, slides were incubated for 10 min at 37°C with horseradish peroxidase (HRP) polymer conjugate (ZYMED; Camarillo, CA). Subsequently, slides were stained with 0.003% DAB and 0.005% hydrogen peroxide in 0.05 M Tris-HCl (pH 7.2), counterstained with hematoxylin, dehydrated, and mounted.

Evaluation of Immunohistochemical Staining

Positive reactions were defined as those showing brown signals in the cell cytoplasm. Each separate tissue core was scored on the basis of the intensity and area of positive staining. The intensity of positive staining was scored as follows: 0, negative; 1, weak staining; 2, moderate staining; 3, strong staining. The rate of positive cells was scored on a 0–4 scale as follows: 0, 0–5%; 1, 6–25%; 2, 26–50%; 3, 51–75%; 4, >75%. If the positive staining was homogeneous, a final score was achieved by multiplication of the two scores, producing a total range of 0–12. When the staining was heterogeneous, we scored it as follows: each component was scored independently and summed for the results. For example, a specimen containing 25% tumor cells with moderate intensity ($1 \times 2 = 2$), 25% tumor cells with weak intensity ($1 \times 1 = 1$), and 50% tumor cells without immunoreactivity received a final score of $2 + 1 + 0 = 3$. For statistical analysis, we grouped all the samples into two groups, according to the positive expression: scores of 0–8 as low expression and scores of 9–12 as high expression.

We selected the higher scores of fascin and the corresponding tissue core scores of phospho-S39-fascin (Figure SF3).

Western Blot Analysis

Tumor tissues and cells were lysed in Phosphosafe Extraction Reagent (EMD Biosciences; San Diego, CA). The lysates were then centrifuged for 5 min ($16,000 \times g$, 4°C). The protein concentration was estimated by the Bradford method. Equal amounts of tissue or cell lysates (100 μ g) were electrophoresed on 12% polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore; Bedford, MA). The membranes were then blocked with 5% skim milk–PBS Tween (0.01 M

PBS, 0.05% Tween 20) for 1 hr and incubated at room temperature for 2 hr with the primary antibodies as described earlier. The membranes were subsequently incubated at room temperature for 1 hr with HRP-linked secondary antibodies and analyzed using Western Blotting Luminol Reagent (Santa Cruz Biotechnology; San Diego, CA). Image acquisition and quantitative analysis were carried out using the FluorChem 8900 image analysis system (Alpha Innotech; St San Leandro, CA).

Enzyme-linked Immunosorbent Assay

Microtiter plates (96-well) were incubated with 0.2 μ g/well purified peptide or control peptide overnight at 4°C. After washing four times with 0.05% Tween 20 in PBS to remove unbound peptide, plates were blocked with 1% BSA in carbonate sodium buffer (0.05 M, pH 9.6). Next, purified rabbit antibody or preimmune sera or immune sera targeted against the peptides were added at various dilutions for 1 hr at 37°C. Plates were washed four times with 0.05% Tween 20 in PBS and then incubated for 40 min with goat anti-rabbit/mouse IgG coupled to HRP (1:2000 in PBS). After washing with 0.05% Tween 20 in PBS, 100 μ l of the colorimetric HRP substrate solution (TMB; BETHYL, Montgomery, TX) was added to each well. After 8 min, the reaction was stopped with 100 μ l of 2 M H₂SO₄, and the plates were read at 450 nm in a microplate reader.

Statistical Analysis

Pearson's χ^2 test with SPSS 13.0 was used to evaluate the association between fascin and phospho-S39-fascin expression and clinicopathological factors such as age, gender, tumor location, tumor size, histological grade, primary tumor, regional lymph nodes, and distant metastasis. The overall survival was defined as the time from the date of operation to the date of death due to cancer. The Kaplan–Meier method with log-rank test was used to analyze the survival conditions of the patients with ESCC. Multivariate analysis was performed using the Cox regression model to study the effects of different variables on the overall survival. We selected gender, age, and the factors within a significant level of 0.10 in univariate analysis for a forward stepwise regression model. However, pTNM stage was excluded because of its correlation with extent of tumor, regional lymph nodes, and distant metastasis in the model. *p* Values less than 0.05 were considered significant.

Results

Specificity Characterization of the Custom Rabbit Anti-human Phospho-S39-fascin Antibody

To investigate the specificity of the custom rabbit anti-human fascin antibody, ELISA and immunohistochemical experiments were performed (Figures 1, SF1,

and SF2). ELISA experiments showed that immune serum specifically recognized immobilized immunizing peptide compared with preimmune serum (Figure SF1A) and that the purified custom rabbit anti-human phospho-S39-fascin antibody specifically detected immobilized immunizing peptide [VNASAS(p-S)LKK] compared with mouse anti-human fascin antibody (Figure SF1C). Furthermore, the custom rabbit anti-human phospho-S39-fascin antibody had no cross-reactivity with control peptide (Figure SF1B) and fascin peptide (VNASASSLKK) (Figure SF1D). Immunizing peptide adsorption experiments by immunohistochemical method showed that no immunoreactivity or weak immunoreactivity was observed when phospho-S39-fascin antibody was replaced with PBS (Figure 1A) or preimmune rabbit serum (Figure 1B). Rabbit anti-human phospho-S39-fascin staining (Figure 1E) was abrogated (Figure 1F) by preadsorption with immunizing peptide [VNASAS(p-S)LKK]. However, no change of mouse anti-human fascin staining was observed either when neutralized with immunizing peptide or when not neutralized (Figures 1C and 1D). In addition, the custom rabbit anti-human phospho-S39-fascin antibody showed remarkably weaker staining (Figures SF2C and SF2D) when treated with alkaline phosphatase. On the contrary, mouse anti-human fascin staining showed no change (Figures SF2A and SF2B). Taken together, these results indicated that rabbit anti-human phospho-S39-fascin antibody specifically recognized the phosphorylation-type protein and that mouse anti-human fascin-1 antibody recognized only the non-phosphorylation-type protein.

Patient Characteristics

A total of 254 ESCC specimens were examined by immunohistochemical staining. Statistical analysis showed that there was no survival advantage with the use of radiotherapy or chemotherapy compared with the surgery-alone group, so we analyzed the patients' survival together. The 5-year overall survival rate of the 254 patients was 40.1%. Kaplan–Meier survival method revealed that the prognosis of patients was significantly associated with tumor size, histological grade, regional lymph node metastasis, distant metastasis, and pTNM stage (*p* < 0.05) (Table 1).

Expression of Fascin and Phospho-S39-fascin in ESCC Samples and Cell Lines

Both fascin and phospho-S39-fascin were predominantly expressed in cytoplasm (Figure 2). Immunoreactivity was also observed in endothelial cells and stromal cells in the underlying lamina propria. Positive staining of fascin was apparent only in basal and lower spinous layer of the normal epithelium (Figure 2A) but diffusely in ESCC tissues, and almost all the specimens showed homogeneous staining (Figures 2B and 2D).

Figure 1 Characterization of the custom rabbit anti-human phospho-S39-fascin antibody by immunizing peptide adsorption experiments using immunohistochemical methods. Negative or weak immunoreactivity was observed when phospho-S39-fascin antibody was replaced with PBS (A) or preimmune rabbit serum (B). Mouse anti-fascin staining showed no change whether neutralized with immunizing peptide [VNASAS(p-S)LKK] (C) or not (D). On the contrary, rabbit anti-human phospho-S39-fascin staining (E) was abrogated (F) by preadsorption with immunizing peptide. Bar = 50 μ m.

In cancerous tissue, tumor cells exhibited moderate-to-intense diffuse labeling by fascin, infiltrative margins showed stronger immunoreactivity, and the squamous pearl was negative for fascin. Phospho-S39-fascin expression showed a little difference. In normal epithelium, phospho-S39-fascin was negative or expressed to various degrees (Figure 2E). In ESCC, stronger immunoreactivity of the infiltrative margins was not observed (Figures 2G and 2H).

Statistical analysis showed a positive correlation of fascin immunostaining with tumor size ($p=0.001$). High expression of fascin was present in only 33 of 139 cases (26.6%) with tumors less than 5 cm in diameter, but it was present in 53 of 115 cases (46.1%) with tumors of diameters greater than or equal to 5 cm. Other factors, such as age, gender, regional lymph nodes, distant metastasis, and tumor location, however, had no significant association with fascin expression (Table ST1). The correlations between phospho-S39-fascin protein expression and various clinical factors

were investigated, but no significant correlation was found (Table ST2).

To confirm the immunohistochemical results of fascin and phospho-S39-fascin expression, we then performed a Western blot analysis in cells and samples. High expression of fascin was detected in all ESCC cell lines tested. However, the expression of phospho-S39-fascin was at different levels in cell lines. Phospho-S39-fascin was very weak or absent in most cell lines except for KYSE150 and EC109 (Figure 3A). The results of the Western blot analysis in tissue samples showed that higher expression of fascin was detected in tumor tissues compared with corresponding normal epithelium, and phospho-S39-fascin was detected at an equal or higher level in normal tissues than in ESCC (Figure 3B).

High Expression of Fascin in Patients With ESCC Was Associated With Poor Survival

Calculations of survival by the Kaplan–Meier method revealed that fascin expression was a significant prognostic

Figure 2 Immunohistochemical staining of fascin and phospho-S39-fascin in normal esophagus and esophageal squamous cell carcinoma (ESCC). Phospho-S39-fascin (E) showed stronger immunoreactivity in normal epithelium than fascin (A). The region with strong positive immunoreactivity of fascin (B) showed weak positive immunoreactivity for phospho-S39-fascin (F) in the same tumor. On the contrary, the region with negative immunoreactivity of fascin (C) showed strong positive immunoreactivity for phospho-S39-fascin (G) in the same tumor. In another pair of samples (D,H), both fascin and phospho-S39-fascin exhibited strong positive immunoreactivity. Bar = 50 μ m.

factor. Stronger fascin immunoreactivity correlated with a poor prognosis. Patients with fascin high-expression tumors had a 5-year survival rate of 34.5%, compared with 43.0% for patients with fascin low-expression tumors ($p=0.006$; Figure 4A). Moreover, the Cox multivariate analysis showed that fascin expression was a negative independent poor prognostic factor for overall survival and that the risk ratio for patients with high expression of fascin was 1.604 with

a 95% confidence interval (CI) of 1.145–2.248 ($p=0.006$; Table 2).

High Expression of Phospho-S39-fascin in ESCC Was Associated With Improved Prognosis

Survival analysis by the Kaplan–Meier method revealed that phospho-S39-fascin expression was a significant prognostic factor. As mentioned earlier, high

Figure 3 Western blot analysis of fascin and phospho-S39-fascin expressions in cell lines and representative tissue samples. **(A)** High expression of fascin was detected in all the ESCC cell lines, whereas phospho-S39-fascin was weak or absent in most cell lines except for KYSE150 and EC109. **(B)** Fascin often showed a higher level in the tumor tissue than corresponding normal epithelial tissue. Phospho-S39-fascin was examined at an equal or higher level in normal tissue than in ESCC. Detection of β -actin was used as a loading control.

expression of fascin was associated with poor survival in patients with ESCC. Interestingly, the immunoreactivity of phospho-S39-fascin was the exact opposite of that. Patients with phospho-S39-fascin high-expression tumors had a 5-year overall survival rate of 50.2%, compared with 35.2% for patients with phospho-S39-fascin low-expression tumors ($p=0.016$; Figure 4B). The Cox multivariate analysis showed that high expression of phospho-S39-fascin was an independent positive prognostic factor. The risk ratio was 0.661 with a 95% CI of 0.455–0.960 ($p=0.030$; Table 2).

Fascin/Phospho-S39-fascin Expression and Patients' Outcome

The 254 patients were divided into four groups based on the fascin and phospho-S39-fascin immunoreactivity (Table 3; Figure SF4). Statistical analysis revealed that the overall survival rates of the groups were significantly different ($p=0.003$; Tables 3 and ST3). Patients ($n=58$) with fascin^{low}/phospho-S39-fascin^{high} tumors had the longest survival with a 5-year overall survival rate of 51.5%. On the contrary, patients ($n=61$) with fascin^{high}/phospho-S39-fascin^{low} tumors had the worst prognosis among these four groups with a 5-year overall survival rate of 28.7%. As compared with the two groups mentioned earlier, survival of the other two groups (patients with fascin^{high}/phospho-S39-fascin^{high} tumors and patients with fascin^{low}/phospho-S39-fascin^{low} tumors) was between those two groups. Interestingly, patients with fascin high-expression tumors could also have a relatively better prognosis when phospho-S39-fascin had strong immunoreactivity. As mentioned earlier, we found that high expression

Figure 4 Overall survival of 254 patients with ESCC vs fascin **(A)** and phospho-S39-fascin **(B)** expressions. Survival rates were analyzed using the Kaplan-Meier survival test. **(A)** Higher fascin immunoreactivity was associated with worse survival of patients ($p=0.006$). **(B)** Prognosis of patients with high expression of phospho-S39-fascin was better when compared with those with low expression of phospho-S39-fascin ($p=0.016$).

of fascin was associated with poor survival (5-year overall survival rate was 34.5%) of patients, but when considering phospho-S39-fascin expression together, the two subgroups had an obviously different survival rate. Patients with high expression of phospho-S39-fascin (29 of 90 patients) had a 5-year overall survival rate of 48.3%, compared with 28.7% for patients with low expression of phospho-S39-fascin (61 of 90 patients), though it did not reach statistical significance ($p=0.068$; Figure 5).

Table 2 Cox multivariate regression analysis for predicting overall survival

	Risk ratio	95% CI of RR	p Value*
Regional lymph nodes (N1)	2.628	1.865–3.703	0.000
Fascin (high)	1.604	1.145–2.248	0.006
Regional lymph nodes (N1)	2.584	1.832–3.646	0.000
Phospho-S39-fascin (high)	0.661	0.455–0.960	0.03
Regional lymph nodes (N1)	2.619	1.857–3.693	0.000
Fascin ^{high} /phospho-S39-fascin ^{low}	1.854	1.296–2.654	0.001

*p Values <0.05 were considered significant.

CI, confidence interval; RR, risk ratio; low, low expression (scores of 0–8); high, high expression (scores of 9–12).

Multivariate analysis (including gender, age, histological grade, extent of tumor, regional lymph nodes, distant metastasis, tumor size, and fascin^{high}/phospho-S39-fascin^{low} expression) showed that regional lymph nodes (N1, $p=0.000$) and fascin^{high}/phospho-S39-fascin^{low} expression ($p=0.001$) were independent prognostic factors (Table 2).

Discussion

ESCC is a lethal malignancy, and development of biomarkers for predicting prognosis is an urgent need. The actin-bundling protein fascin is one of the potential candidates of predictor. It has been reported that high expression of fascin is associated with poor survival in patients with ESCC by influencing cell proliferation and invasiveness through participation in the formation of cell protrusions (Hashimoto et al. 2005; Xie et al. 2005; Xue et al. 2006). Previous studies have shown that phospho-S39-fascin has a dominant negative effect on the filopodia formation (Yamakita et al. 1996; Adams et al. 1999; Vignjevic et al. 2006; Aratyn et al. 2007); however, no research about the assessment of phosphorylated fascin protein in tumors has been reported.

In our study, we first evaluated fascin and phospho-S39-fascin expressions in ESCC and the matched distal resected margin samples by IHC. We found that both fascin and phospho-S39-fascin were predominantly expressed in cytoplasm, and invasive margins of cancer nests showed stronger immunoreactivity of fascin.

Table 3 Association between fascin and phospho-S39-fascin status and patient prognosis

Fascin/phospho-S39-fascin expression	No. of cases	5-Year overall survival rate (%)
Fascin ^{low} /phospho-S39-fascin ^{high}	58	51.5
Fascin ^{low} /phospho-S39-fascin ^{low}	106	38.5
Fascin ^{high} /phospho-S39-fascin ^{high}	29	48.3
Fascin ^{high} /phospho-S39-fascin ^{low}	61	28.7

Low, low expression (scores of 0–8); high, high expression (scores of 9–12).

Figure 5 Kaplan-Meier survival curves for patients with tumors of high expression of fascin. Those patients with high expression of phospho-S39-fascin showed a better survival rate, though it did not reach the statistical significance ($p=0.068$).

Normal esophagus usually showed weak immunoreactivity of fascin and negative or positive to various degrees of immunoreactivity of phospho-S39-fascin. Our results of Western blot analysis accorded with the immunohistochemical method. ESCC cells and samples showed higher levels of fascin expression but lower or equal levels of phospho-S39-fascin expression than normal cells. Survival analysis showed that the phosphorylated or non-phosphorylated state of fascin had opposite effects on patients' prognosis. High expression of fascin correlated with poor survival, and high expression of phospho-S39-fascin correlated with improved survival in patients with ESCC.

Previous studies showed that fascin was localized in the cytoplasm, especially in the membrane protrusions of ESCC cells (Xie et al. 2005; Zhang et al. 2006). In contrast, phosphorylated fascin was primarily freely diffusing and weakly localized to filopodia. It has been proposed that fascin was present in the cytoplasm as a mixture of inactive phosphorylated and active non-phosphorylated forms. Activation of fascin by nonphosphorylation allowed for high-affinity actin binding in filopodia, and inactivation of fascin by phosphorylation inhibited filopodia formation, cell migration, tumor development, and metastasis. However, phosphorylated fascin was not excluded from filopodia. The dominant negative effect prominently altered the filopodia formation and played a role in the structural organization (Yamashiro et al. 1998; Vignjevic et al. 2006; Aratyn et al. 2007; Hashimoto et al. 2007). The phosphorylation status was regulated by cell-matrix adhesions through related pathways

(Adams et al. 1999; Hashimoto et al. 2007). Combined with our data, these results suggest that fascin may undergo a disturbed dynamic balance between phosphorylated and non-phosphorylated status during the progression of normal esophagus to ESCC. Fascin was mainly present as inactive phosphorylated status at a low level in normal esophagus, whereas in ESCCs, upregulated fascin was activated by dephosphorylation at various degrees. The imbalance of fascin phosphorylation/dephosphorylation may play an important role in the progression of ESCC. Downregulation of phosphorylated fascin or upregulation of non-phosphorylated fascin caused invasive behavior in esophageal epithelial cells by participating in filopodia formation as mentioned earlier. According to our data, fascin expressed at a high level and correlated with poor survival in patients with ESCC, and phospho-S39-fascin expressed to various degrees in normal esophagus and its high expression correlated with improved survival in patients with ESCC. Even the patients with tumors of high expression of fascin could have a relatively better prognosis when phospho-S39-fascin had strong immunoreactivity. This suggests that the reduced risk of poor survival of patients by high expression of phospho-S39-fascin could result from a reduced proliferation and invasive properties of tumor cells, based on the findings mentioned previously (Yamashiro et al. 1998; Xie et al. 2005; Vignjevic et al. 2006). Unfortunately, significant association between phospho-S39-fascin expression and regional lymph node metastasis was not found in this study ($p > 0.05$), suggesting that other unknown mechanisms may be involved in this fascin-mediated progression. Therefore, an animal model expressing phosphomimetic fascin mutants and further investigations are needed in future studies.

In summary, both fascin and phospho-S39-fascin expressions were independent prognostic factors. High expression of fascin was associated with poor overall survival, which confirmed the previous studies, and high expression of phospho-S39-fascin improved the overall survival in patients with ESCC. This suggests that the dynamic balance between phosphorylation and non-phosphorylation status of fascin may be associated with the ESCC progression and has an important role in the prognosis of patients with ESCC. Inhibiting the non-phosphorylation level of fascin or inducing its phosphorylation level could decrease the risk of poor prognosis. Fascin and phospho-S39-fascin may be attractive therapeutic strategies for ESCC in future clinical trials and candidates for prediction of patients' prognosis.

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