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*Original Article*

# Fibronectin and laminin are related to malignant properties of tumor through affecting mesenchyme in esophageal squamous cell carcinoma

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**Abstract:** Non-collagenous extracellular matrix fibronectin (FN) and laminin (LN) located on the epithelial basement membranes were deemed to prevent tumor cells from invading interstitial substance. Whereas emerging evidences bring to our attention in the process their crucial roles of promoting evolution in several tumors. We investigated the fibronectin and laminin expression in esophageal squamous cell carcinoma (ESCC) tissues, as well as their associations with the densities of microvessels and stromal myofibroblasts (MFs) in tumor mesenchyme, finally analyzed their clinical significance. 53 tumor specimens were collected. Immunohistochemistry was performed to detect the levels of fibronectin and laminin expression in tumor tissues, and the densities of microvessels and stromal myofibroblasts in tumor mesenchyme. The fibronectin and laminin expression in ESCC tissues were conspicuously abundant. Fibronectin upregulation was proportionate to high microvessels density (MVD) ($P = 0.008$) and plentiful stromal myofibroblasts ($P = 0.016$). Analogously, laminin expression had positive relationships with MVD ($P = 0.039$) and quantity of stromal myofibroblasts ($P = 0.023$). Their respective associations with TNM stage (FN: $P = 0.000$, LN: $P = 0.004$) and venous invasion (FN: $P = 0.002$, LN: $P = 0.011$) were verified to be intimate. Moreover, survival analyses revealed statistical discrepancies between outcomes of the patients with negative/weak/moderate and strong expression patterns of both fibronectin and laminin, however their values of predicting prognosis independently were skeptical. We proposed that the upregulated fibronectin and laminin in ESCC tissues were related to the malignant properties of tumor through establishing a permissive environment for tumor invasion and metastasis.

**Keywords:** Esophageal squamous cell carcinoma, fibronectin, laminin, microvessels density, myofibroblasts

## Introduction

Esophageal squamous cell carcinoma (ESCC) is relatively common among digestive tract cancers in China. Early metastasis and recurrence make it tough to prolong patients' survival even with multidisciplinary treatments. From macroscopic view, typical anatomical features of esophagus, distribution of aplenty lymph nodes, and the vascular networks connecting to neck and chest contribute to tumor local invasion and distant metastasis [1], of which more essential molecular mechanisms have not been fully understood. Therefore, identification of targets involved in ESCC invasion and metastasis is significant in improving novel therapeutic strategies.

Fibronectin (FN) and laminin (LN) as important extracellular matrix glycoproteins, were previously researched about their barrier action of assembling completed sheet-like structures located on the epithelial basement membranes in normal tissues, where breaches and cleavages of fibronectin and laminin expression were found in the terminal ESCC tissues [2]. Several lines of evidences indicated different isoforms of fibronectin and laminin widely distributed in the tumor tissue had pleiotropic functions during tumor evolution including inducing apoptosis in breast and oesophageal cancer cells [3], facilitating tumor invasion and metastasis by interacting with the receptor integrins in glioblastomas, cervical, breast and ovarian cancer [4-7], and participating in tumor angiogenic

modulation and epithelial-mesenchymal interaction [8, 9].

Functional researches of fibronectin and laminin in vascular system derived from in-vitro and in-vivo models focused on the reconstruction of vascular basement membrane and the interaction with vascular endothelial cells. For instance, fibronectin was secreted as new matrix of vascular basement membrane extensively remodeled during tumor angiogenesis [10], while laminin could affect endothelial cells through favoring proliferation and capillary-like structured formation [11], and was proved to promote tube formation, a measure of angiogenesis in trophoblast cells [12]. Remodeling of fibronectin and laminin involved in tumor angiogenesis has been reported in colorectal carcinoma, glioblastoma and breast cancer [13-15]. Cancer associated fibroblasts (CAFs) with a phenotype similar to myofibroblasts (MFs) are widely known to synergize with neoplastic cells from tumor initiation to evolution, in which fibronectin fibril assembly was found to be increased by promotion of neuropilin-1 [16]. Besides mesenchyme, in the cancer cells co-cultured with CAFs fibronectin upregulation was also observed such as human tongue cancer [17]. Accordingly, laminin expression was verified to be associated with the density of stromal myofibroblasts in oral squamous cell carcinoma and with the designated interface zone-fibroblasts in breast cancer [18, 19]. ESCC was deemed to possess a characteristic that the invasion and metastasis of tumor perceived enriched intratumoral microvessels and stromal fibroblasts as essential, nevertheless rarely have the investigations of fibronectin and laminin focused on their relationships with tumor angiogenesis and myofibroblasts been carried out in ESCC.

In the current work we employed immunohistochemistry to investigate the influences of fibronectin and laminin in ESCC tissue upon tumor mesenchyme, and analyzed their associations with clinical significance. We found that the overexpression of fibronectin and laminin in tumor tissue were related to the malignant properties of tumor and worse outcomes of patients in ESCC, and the remodeling of tumor mesenchyme that inclined cancer cells towards invading and migrating might be considered as one valid approach.

## Materials and methods

### *Patients and tumor biopsies*

Fifty-three patients with ESCC at a median age of 65 years old (range 40-82) were selected who underwent curative transthoracic esophagectomy at the Department of Thoracic Surgery, from Jan 2007 to Jan 2008. All cases were diagnosed on a clinical basis with pathological confirmation and no patients received additional treatment prior to the operation. The clinicopathological data collected included ≥ 60 (n = 32), ≤ 60 (n = 21), male (n = 39), female (n = 14), poor differentiation (n = 17), moderate and well differentiation (n = 36), TNM I/II (n = 26), TNM III/IV (n = 27), lymphatic metastasis (n = 23), and vascular invasion (n = 29). TNM stage was followed with the NCCN Clinical Practice Guidelines in Oncology Version 2009. All patients' tissue specimens were 10% formalin-fixed, paraffin-embedded and hematoxylin eosin staining for immunohistochemistry. Clinicopathological data of all patients were obtained from medical records and follow-ups with a follow-up deadline of Sep 17, 2015. This study was approved by the Institutional Review Board of the Anhui Provincial Hospital affiliated to Anhui Medical University and written consent was obtained from all participants.

### *Immunohistochemistry and evaluation*

The expressions of fibronectin, laminin, CD34, and α-smooth muscle actin (α-SMA) in ESCC and adjacent normal tissues were detected by immunohistochemistry using a two-step method (Zhongshan Jinqiao Co., Beijing, China). Four-micrometer-thick paraffin slices marked with fibronectin, CD34, and α-SMA were dewaxed to water flushing process then boiled with high pressure for 2 min immersed in citrate-buffer (10 mmol/L pH=6.0) for antigen repairment. 3% hydrogen peroxide was used to block the endogenous peroxidase activity. Specimen slices marked with laminin were treated by trypsin (1:5 Zhongshan Jinqiao Co., Beijing, China) in water bath heating at 37°C for 10 min to repair antigen. After washing by phosphate balanced solution (PBS), sections were respectively incubated with primary rabbit anti-human fibronectin polyclonal antibody (1:100, Zhongshan Jinqiao Co., Beijing, China), mouse anti-human laminin monoclonal antibody (1:50,

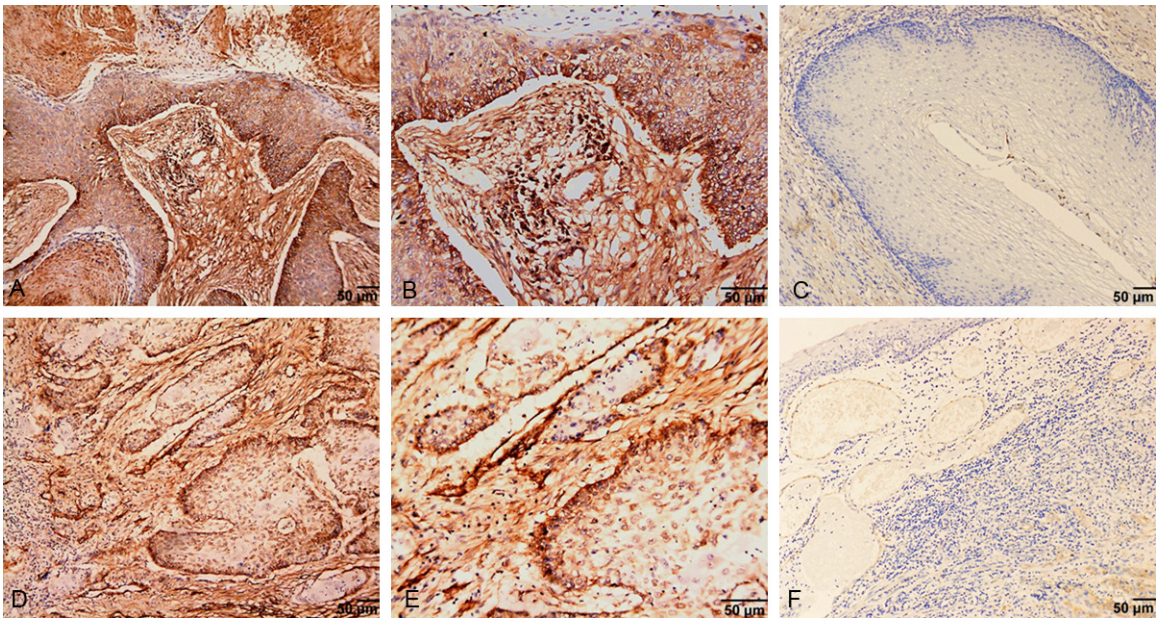


**Figure 1.** Immunohistochemistry showed expression diversities of the fibronectin and lamininin in tumor and paracarcinomatous tissue of ESCC. Expressions of fibronectin (A: 100 ×, B: 200 ×) and laminin (D: 100 ×, E: 200 ×) in the ESCC tissue were detected to be conspicuously stronger than that in the paracarcinomatous tissue (C: FN, 100 ×, F: LN, 100 ×).

Zhongshan Jinqiao Co., Beijing, China), mouse anti-human CD34 monoclonal antibody (1:100, Zhongshan Jinqiao Co., Beijing, China), and mouse anti-human α-SMA monoclonal antibody (1:100, Zhongshan Jinqiao Co., Beijing, China) at 4°C overnight. After incubating with the labeled secondary mouse anti-rabbit antibody (Zhongshan Jinqiao Co., Beijing, China) at room temperature for 30 min, the sections were colored through reacting with 3, 3-diaminobenzidine (DAB; Zhongshan Jinqiao Co., Beijing, China) and hematoxylin counter staining, finally were dehydrated, transparentized, and slides-integrated. Negative control slides were processed with PBS and a known positive tissue slice was used as a positive control.

The semi-quantitative integration was used to evaluate expression levels of fibronectin and laminin in the tumor cells and stroma. The yellow-brown granules located in cytoplasm, cytomembrane, and stroma were defined as positive staining. Staining intensity was scored as follows: 0, negative; 1, yellow; 2, brown; and 3, sepia. Actual background should be taken for reference in staining practice. In regard of fibronectin and laminin in the epithelial cells, scores for the average percentage of immunopositive cells were based on each 100 cells in the 10 high-power fields (400 ×) from the "hot spots" in low power fields (100 ×), and rated as follows: 0, < 10% positive cells; 1, 10%-25%; 2, 25%-50%; 3, 50%-75%; and 4, > 75% positive cells. While the stromal positivity of fibronectin and laminin were assessed in low power field (100 ×) by the analogous scoring system: 0, < 10% positive stromal compartment; 1, 10%-25%; 2, 25%-50%; 3, 50%-75%; and 4, > 75% positive stromal compartment. We averaged the products respectively from epithelial cell and stroma, which were of the scores based on staining intensity and immunopositive ratio, to evaluate the staining status of ESCC and paracarcinomatous tissue according to the criterion as follows: 0, negative (-); 0-4 (including 4), weak positive (+); 4-8 (including 8), moderate positive (++); and 8-12, strong positive (+++).

Immunohistochemical level assessment of the stromal positivity of α-SMA in low power field (100 ×) was semi-quantitatively followed the scoring system below: (-), no stromal staining; (+), weak staining in a few stromal areas; (++), moderate staining of the stromal compartment; and (+++), strong staining in the majority of the stromal compartment. MVD evaluation was acted in accordance with the following principles. 3-5 "hot spots" were selected in low power fields (100 ×) in which intratumoral microvessels distributed densely, amounts of

**Table 1.** Positive rates of fibronectin and laminin expression in the tumor and paracarcinomatous tissues of ESCC

| | Tumor tissues | Paracarcinomatous tissues | $X^2$ | *P* |
|---|---|---|---|---|
| FN | 51/53 (96.2%) | 34/53 (64.2%) | 17.162 | 0.000 |
| LN | 28/53 (52.8%) | 5/53 (9.4%) | 23.277 | 0.000 |

FN: fibronectin, LN: laminin.

**Table 2.** Associations of the expressions of fibronectin and laminin in ESCC tissues with the densities of intratumoral microvessels and myofibroblasts

| | Density of microvessels | | Density of myofibroblasts | |
|---|---|---|---|---|
| | r | *P* | r | *P* |
| FN | 0.361 | 0.008 | 0.330 | 0.016 |
| LN | 0.284 | 0.039 | 0.311 | 0.023 |

FN: fibronectin, LN: laminin.

vessels were quantified in the 1-3 high-power fields (400 ×) from every "hot spot" depending on its size. Rank assessment of the average amount was followed by the criterion below: less than 10, weak positive (+); 10-30, moderate positive (++); and more than 30, strong positive (+++).

All slices were assessed by two pathologists who were blind to diagnosis independently.

*Statistical analysis*

Statistical analysis was completed with SPSS 13.0 (SPSS, Inc., Chicago, IL) and $P < 0.05$ was considered statistically significant. The Chi-square test was used for comparison of positive ratio. Associations of the fibronectin and laminin in tumor tissue with the densities of microvessels and myofibroblasts in tumor stroma were performed by Spearman's rank correlation test. Whether there existed statistical discrepancies between outcomes of patients with poor and strong expression patterns of fibronectin and laminin was determined by Kaplan-Meier analysis and log-rank test. The Cox regression model was used for multivariant survival analysis.

## Results

*Expressions of fibronectin and laminin in the ESCC tissues*

Fibronectin and laminin expression were observed to be abundant in the cytoplasm and cytomembrane of ESCC cells especially the tumor marginal cells, namely so-called invasion front, as well as in the surrounding submucosal mesenchymal tissues. **Figure 1** displayed two representative slices in immunohistochemistry.

Positive rate of fibronectin in the ESCC tissues was 51/53 (96.2%), conspicuously higher than that in the paracarcinomatous tissues (34/53, 64.2%, $X^2 = 17.162$, $P = 0.000$). Analogously, compared to adjacent normal tissues where 5/53 (9.4%) of the cases were laminin positive at the low level, 28/53 (52.8%, $X^2 = 23.277$, $P = 0.000$) of the ESCC tissue specimens expressed laminin (**Table 1**).

*Associations of the expressions of fibronectin and laminin in ESCC tissues with the densities of microvessels and myofibroblasts in tumor stroma*

ESCC tissue was affluent with microvessels and myofibroblasts visualized by CD34 and α-SMA in general. Spearman's correlation test revealed that the fibronectin expression in ESCC tissue was positively related to intratumoral MVD ($r = 0.361$, $P = 0.008$) and quantity of stromal myofibroblasts ($r = 0.330$, $P = 0.016$). Additionally, the associations of the laminin in ESCC tissue with intratumoral MVD ($r = 0.284$, $P = 0.039$) and quantity of stromal myofibroblasts ($r = 0.311$, $P = 0.023$) were indicated to be significant, as shown in the **Table 2**.

*Clinicopathological significance of the fibronectin and laminin expressed strongly in ESCC tissues*

We employed the Chi-square test to analyze the relationships between clinicopathological features and the strongly positive rates of fibronectin and laminin expression in ESCC tissues. Overexpression of fibronectin was affinitive with TNM stage ($X^2 = 13.867$, $P = 0.000$) and venous invasion ($X^2 = 9.669$, $P = 0.002$), whereas irrelevant to differentiation degree ($X^2 = 3.593$, $P = 0.058$) and lymphatic metastasis ($X^2 = 2.779$, $P = 0.096$). Similarly, there existed intimate associations of laminin upregulation with TNM stage ($X^2 = 8.423$, $P = 0.004$) and venous invasion ($X^2 = 6.512$, $P = 0.011$), however the significant relationships with differentiation degree ($X^2 = 0.251$, $P = 0.616$) and lymphatic metastasis ($X^2 = 0.407$, $P = 0.524$) did not appear. The results above were present in the **Table 3**.

**Table 3.** Clinicopathological significance of the fibronectin and laminin expressed strongly in ESCC tissues

| | | FN | | | LN | | |
|---|---|---|---|---|---|---|---|
| Variable | Cases | Strong positive | $x^2$ | *P* | Strong positive | $x^2$ | *P* |
| TNM stage | | | 13.867 | 0.000 | | 8.423 | 0.004 |
| I/II | 26 | 8/26 (30.8%) | | | 3/26 (11.5%) | | |
| III/IV | 27 | 22/27 (81.5%) | | | 13/27 (48.1%) | | |
| Differentiation degree | | | 3.593 | 0.058 | | 0.251 | 0.616 |
| Low | 11 | 9/11 (81.8%) | | | 4/11 (36.4%) | | |
| High/Moderate | 42 | 21/42 (50.0%) | | | 12/42 (28.6%) | | |
| Venous invasion | | | 9.669 | 0.002 | | 6.512 | 0.011 |
| Positive | 29 | 22/29 (75.9%) | | | 13/29 (44.8%) | | |
| Negative | 24 | 8/24 (33.3%) | | | 3/24 (12.5%) | | |
| Lymphatic metastasis | | | 2.779 | 0.096 | | 0.407 | 0.524 |
| Positive | 23 | 16/23 (69.6%) | | | 8/23 (34.8%) | | |
| Negative | 30 | 14/30 (46.7%) | | | 8/30 (26.7%) | | |

FN: fibronectin, LN: laminin.

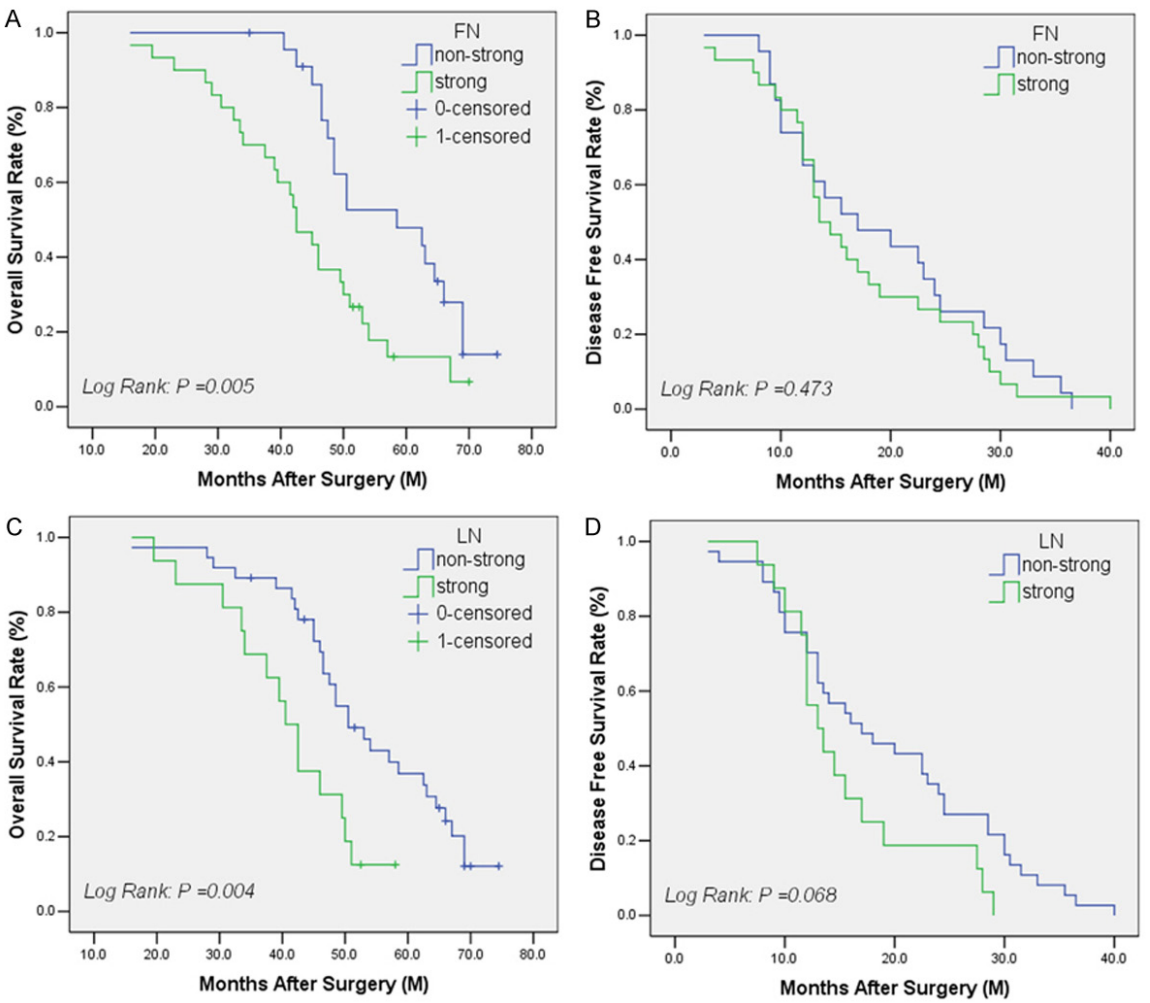


**Figure 2.** Kaplan-Meier analyses of OS and DFS rate curves of the patients with ESCC based on respective expressions of fibronectin and laminin in the tumor tissues as strong and non-strong (negative/weak/moderate). The

overall survivals of patients with non-strong expressions of fibronectin (A) and laminin (C) were significantly longer than those of the patients with strongly expressed levels. Statistical discrepancy between disease free survivals of the patients with strong and non-strong level was found in neither fibronectin (B) nor laminin (D) expression pattern.

**Table 4.** Multivariate analysis of factors associated with overall survival in the ESCC patients

| | | RR 95% CI | |
|---|---|---|---|
| Overall survival | RR | Lower-Upper | *P* |
| FN | | | 0.201 |
| Rich vs Poor | 1.502 | 0.805-2.800 | |
| LN | | | 0.142 |
| Rich vs Poor | 1.228 | 0.934-1.614 | |
| Age | | | 0.697 |
| ≥ 60 vs < 60 | 0.866 | 0.421-1.783 | |
| Gender | | | 0.744 |
| Male vs Female | 1.149 | 0.499-2.644 | |
| TNM stage | | | 0.008 |
| III/IV vs I/II | 3.763 | 1.412-10.028 | |
| Differentiation degree | | | 0.012 |
| High/Moderate vs Low | 0.494 | 0.286-0.854 | |
| Venous invasion | | | 0.755 |
| Negative vs Positive | 0.882 | 0.401-1.940 | |
| Lymphatic metastasis | | | 0.976 |
| Negative vs Positive | 0.987 | 0.428-2.276 | |

FN: fibronectin, LN: laminin, RR: relative risk.

*Survival analysis*

Patients were classified into two groups with strong and non-strong (negative/weak/moderate) level respectively of fibronectin and laminin expression. Univariate survival analyses based on overall survival (OS) and disease free survival (DFS) of ESCC patients were conducted by the Kaplan-Meier method and log-rank test, which exhibited contrast between overall survival rates of the two patients groups in both fibronectin and laminin expressed pattern. Overall survival of the patients with non-strong expression of fibronectin was 57.722±2.468 months, significantly longer than 43.689±2.597 months that the patients had with strongly expressed level ($X^2$ = 7.837, *P* = 0.005) (**Figure 2A**). Statistical discrepancy between overall survivals of the patients with different levels of laminin expression (non-strong: 53.385±2.423 months vs strong: 40.969±2.708 months, $X^2$ = 8.180, *P* = 0.004) was verified to be remarkable (**Figure 2C**).

Remodeling of fibronectin and laminin did not exert influence on the disease free survival of ESCC patients in our samples. Statistical difference between disease free survivals of the two groups of patients was found into neither fibronectin (non-strong: 19.435±1.961 months vs strong: 17.233±1.638 months, $X^2$ = 0.515, *P* = 0.473) nor laminin (non-strong: 28.500±2.504 months vs strong: 17.000±3.031 months, $X^2$ = 3.332, *P* = 0.068) expression pattern (**Figure 2B**, **2D**).

Multivariate survival analysis by the Cox regression model indicated that the overexpression of the fibronectin (RR: 1.502, *P* = 0.201) and laminin (RR: 1.228, *P* = 0.142) in ESCC tissue could not predict patients' overall survival independently. The TNM stage (RR = 3.763, *P* = 0.008) and tumor differentiation degree (RR = 0.494, *P* = 0.012) could be considered as the independent prognostic factors of patients' overall survival in ESCC (**Table 4**).

## Discussions

As main members of the extracellular matrix (ECM), fibronectin and laminin participate in constitution of highly cross-linked interstitial stroma and basement membrane polymers at intratumoral structures, thus provide cancer cells with architectural support, more essentially influence their proliferation, differentiation, polarity and mobility [20]. However, the presence of fibronectin and laminin in the cancer cells should not be excluded because of their intimate interactions with tumor stroma. Conspicuous abundance of fibronectin and laminin in the tumor tissue observed in the current study was consistent with other experimental results in nasopharyngeal carcinoma, bladder cancer, gastric carcinoma cell, and also comprising ESCC [21-24]. Particularly in the ESCC tissue, it has been verified that laminin could provide an autocrine positive-feedback circulation via phosphatidylinositol 3-kinase (PI3K) activation [25], and fibronectin mRNA accumulated in the ESCC cells under epithelial-mesenchymal transition [26], suggesting the potential promotional functions of fibronectin and laminin remodeling in ESCC development.

Our results indicated that the overexpression of fibronectin and laminin in ESCC tissue were

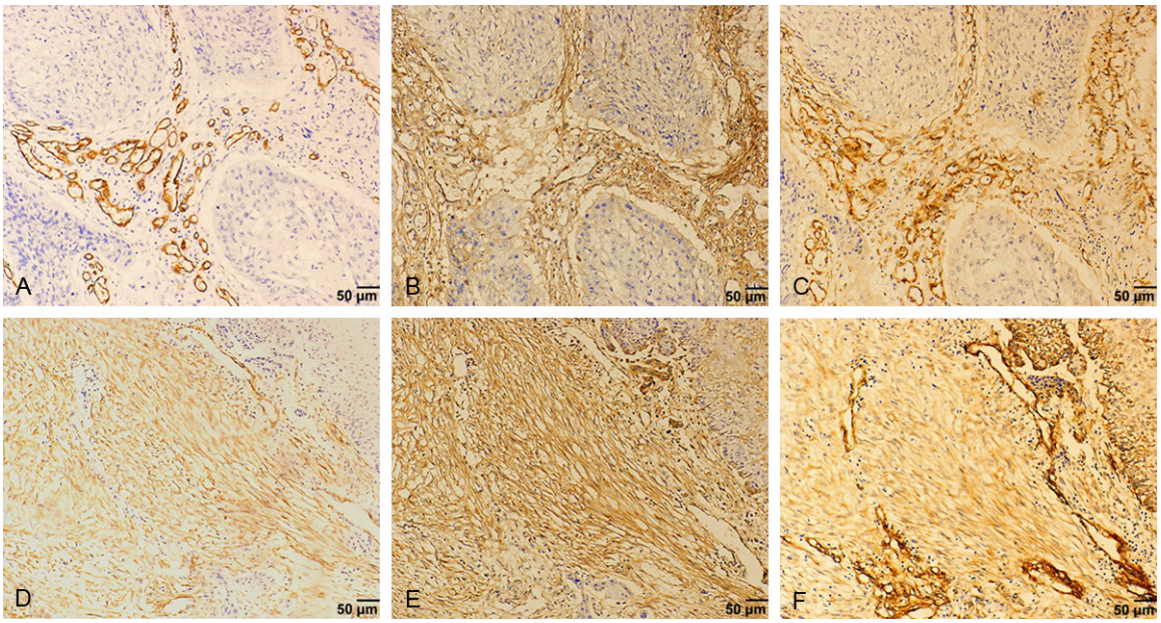


**Figure 3.** Consecutive slides from the same specimen of ESCC tissue exhibited the associations of fibronectin and laminin overexpression with densities of the intratumoral microvessels and myofibroblasts. Fibronectin (B: 100 ×) and laminin (C: 100 ×) increased significantly in the ESCC tissues with plenty of tumor microvessels in malformation (A: 100 ×). Fibronectin (E: 100 ×) and laminin (F: 100 ×) increased significantly in the ESCC tissues with plenty of myofibroblasts (D: 100 ×).

affinitive with higher densities of the tumor microvessels and stromal myofibroblasts. In term of cancer cell, the fibronectin secreted by human oesophageal cancer cell and renal carcinoma cell was found to be involved in modulation of angiogenic micro-environment, particularly in the vascular endothelial growth factor (VEGF)/vascular endothelial growth factor receptor (VEGFR)-2 system [27]. While laminin receptor on the surface of cancer cell was seen to contribute to angiogenesis [28]. Additionally, in oral squamous cell carcinoma, the stroma myofibroblasts were indicated as a preferential source of fibronectin [29]. Laminin upregulation in the myofibroblasts induced by invasive breast cancer cells was demonstrated to confer anoikis-resistant phenotype to the cancer cells [30]. Our experimental data exhibited the epigenetic relationship between deposition of the ECM in ESCC tissue and remodeling of tumor mesenchyme that did point to angiogenesis stimulating and myofibroblast transition (**Figure 3**). Considering angiogenesis as an inherent feature of tumor obtaining conditions for dissemination and growth, alone with the roles of myofibroblasts in stromatogenesis, that could assist in creating a more permissive environment for tumor invasion and metastasis, enrichment of intratumoral microvessels and stromal myofibroblasts were recognized to be associated with malignant properties and poor prognosis in several epithelial tumors [31, 32]. Consequently, we speculated that the overexpression of fibronectin and laminin in ESCC probably result in advanced tumor characteristics through promoting formation of microvessels and transition of CAFs. Actually, biochemical study has showed that the activation of vascular network formation in ESCC needed induction of stromal fibroblasts via the pathways linked to transforming growth factor (TGF)-β and VEGF [33], proposing that the synergy generated by interaction among the intratumoral mesenchymal structures exerted crucial function during tumor evolution. We therefore demonstrated that fibronectin and laminin increased significantly in the ESCC tissues with plenty of tumor microvessels in malformation and myofibroblasts, of which there existed higher proportion of venous invaded and terminal cases. Remodeling was observed to be distributed in cancer cells and submucosal mesenchymal tissues, moreover, marginal cells surrounding tumor nests, namely invasion fronts exhibited extraordinarily strong intensity of expressions, identically expressed feature was reported to be related to advanced grade in the serous epithelial ovarian cancer cells

[34], highlighting the interaction between cancer cells and mesenchyme being able to stimulate synthesis of fibronectin and laminin could promote tumor penetration and invasion.

Regarding as the overexpression of fibronectin and laminin in ESCC tissue conferring the aggressive properties to tumor, we aimed to investigate the clinically relevant findings and found overall survivals of the ESCC patients with fibronectin and laminin strongly positive expression were remarkably shorter than those of the patients with non-strong expressed levels in the univariate analysis. However the disease free survivals of ESCC patients did not varied statistically with different expression patterns of fibronectin and laminin as high and low level, which had nothing in common with some previous researches in ESCC [25, 35], but we further took notice of the individual histological type under observation in each experiment, thus in our study the significant influences on patients' DFS of the fibronectin and laminin increased in ESCC tissue were still arguable. Multivariate survival analysis either failed to indicate the independently predictable values of fibronectin and laminin in the OS of ESCC patients. We envisaged that the present conclusions were preliminary based on insufficient sample amount. Meanwhile, it is needed in order to evaluate the factors influencing prognosis, such as cross-checking on the genetic and transcriptional level, and elaborating observation range.

In conclusion, we revealed that upregulation of the ECM fibronectin and laminin in the ESCC tissue comprising both neoplastic cells and intratumoral mesenchyme, participating in accelerating intratumoral microvessels generation and myofibroblasts transition, could synergize with tumor cells to invade and metastasize thus leading to poor prognosis to an extent. We therefore proposed the potential values of fibronectin and laminin concentrated in the tumor tissue as biomarkers prefiguring early invasion and metastasis of ESCC and blocking targets propitious to ameliorate patients' survival status.

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## Disclosure of conflict of interest

None.

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