Tumor-associated fibroblasts expression in esophageal squamous cell carcinoma and may descend from mesenchymal stem cells

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Research article

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Abstract

Tumor-associated fibroblasts (TAFs) play a pivotal role in cancer proliferation, invasion and metastasis. The expression of TAFs in esophageal squamous cell carcinoma (ESCC) tissues remain unclear. We investigated the relevance of TAFs in ESCC, and then culture mesenchymal stem cells (MSC) by ESCC microenvironment induced their conversion into TAFs to assess whether MSC are precursors of TAFs. We detected the expression of TAFs surface markers, including α-SMA, and S100A4 in esophageal squamous cell cancer tissues by using immunohistochemistry, and evaluated associations with clinicopathological features. In order to verify whether MSC is one of the TAFs sources, we used culture supernatant from ESCC tumor cells to culture and induce MSC to transform into TAFs. TAFs surface markers, α-SMA and S100A4, were expressed in ESCC tissues, and their expressions correlated with tumor differentiation and clinical TNM stage. Real-time-PCR analysis and Western-Blot analysis confirmed MSC conversion into TAFs. Our study demonstrated that TAFs existence in esophageal carcinoma, and their expression was also closely related to metastasis and poor prognosis. Furthermore, we confirmed the conversion from MSC to TAFs and surmised MSC may be the precursors of TAFs.

Introduction

Esophageal squamous cell carcinoma (ESCC) is an aggressive type of epithelial cancer characterized by scarce overall survival [1]. In Asian countries, ESCC accounts for over 90% of esophageal cancers [2] [3]. Apart from the nature inherent to the cancer cells, this poor outcome is also impacted by stromal cells, such as mesenchymal stem cells (MSC) and tumor-associated fibroblasts (TAFs), associated with cancer initiation and progression.

TAFs in cancer stroma, showing characteristics of myofibroblasts with a modified phenotype, play a “pivotal” role in cancer proliferation, invasion and metastasis [4][5]. Common cell surface and well-known markers of TAFs include α-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), Thy–1, S100A4, etc.

Spindle-shaped TAFs secrete growth factors which in turn alter the extracellular matrix (ECM) to create a tumor niche and enhance tumorigenesis, tumor cell proliferation, migration and thus cancer metastasis. Tumor progression is partly a result of evolving crosstalk between different cell types within the tumor and its surrounding supportive tissue or tumor stroma [6]. Several *in vitro* studies in ESCC cell lines have reported the dependency of cancer cell proliferation, angiogenesis, and mobility on the presence of activated fibroblasts [7].

Common cell surface markers of TAF include among others, α-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), Thy–1 and S100A4. The expression levels of these markers are different in various tumor tissues, and α-SMA is usually used to act as an important marker of TAF. [8]

Our study assessed the expression of TAFs markers α-SMA and S100A in ESCC tissues using immunohistochemistry (IHC). They aim was to evaluate their relationship with patients

clinicopathological features and look into the clinical relevance of TAFs in ESCC. Furthermore, we simulated tumor microenvironment using ESCC cells culture supernatant (Eca–109 and TE–1 cells), to culture mesenchymal stem cells (MSC) and induce their conversion into TAFs, to observe the behaviour and expression of α-SMA and S100A during the conversion and assess whether MSC are precursors of TAFs.

Materials And Methods

1. TAF expression in esophageal squamous cell cancer tissues

Patients and cell strains

This study involved 68 patients (54 males and 14 females) who underwent radical esophagectomy for pathologically confirmed esophageal squamous cell carcinoma, in the Department of Thoracic Surgery of the First Affiliated Hospital of Sun Yat-Sen University from September 2005 to March 2010. All eligible, consecutive cases during this time period were identified for this study. All patients signed an informed consent before esophagectomy, and another informed consent was obtained from them prior to collecting and using the specimens. This study received the approval of the Institutional Research Ethics Committee of the First Affiliated Hospital of Sun Yat-Sen University.

Immunohistochemical staining

Esophageal cancer sections were heated at 65°C for 2–3 hours and deparaffinized with xylene. They were then hydrated using 100%, 95%, 85%, and 70% graded alcohol series (five minutes each) and immersed for five minutes in deionized water. After antigen retrieval followed by heat mediated antigen retrieval, the sections were cooled at room temperature and soaked into 0.01M PBS for 3×5 min. Drops of 0.3% hydrogen peroxide were added to cover the whole section, followed by incubation (room temperature for 20 min). The sections were rinsed 3×5 min in 0.01M PBS, blocked by normal goat serum for 10 min, dripped with corresponding primary antibodies (α-SMA at 1:800 and S100A4 at 1:800), and incubated overnight at 4 °C. After 30 min cooling at room temperature, the section were dipped in 0.01M PBS (3 changes of 5 min each), dripped and covered with secondary antibodies and incubated (room temperature for 30 min). Later, they were rinsed with 0.01M PBS (3 changes of 5 min each), dripped with tertiary antibodies, and washed again with 0.01M PBS 3×5 min after incubation (room temperature for 10min). After soaking the sections in deionized water, the sections were treated with DAB, counterstained with hematoxylin before being dehydrated in graded ethanol (70%, 85%, 95%, 100%) and mounted on neutral resin for microscopic observation and photography.

Qualitative appreciation of the immunohistochemical staining for α-SMA and S100A4 was performed by assigning a score according to the staining intensity, where 3 is strong staining, 2 moderate staining, 1 weak staining and 0 is no staining. Quantitatively, after choosing 12 non-overlapping random visual fields

on the stained slide at x400 magnification, the total number of stromal fibroblasts was counted as well as the total number of stromal fibroblasts for every staining intensity. The final immunohistochemistry score was calculated using the formula

$$Immunohistochemical\ score = \sum_{k=0}^{3}[k \times (Percentage\ of\ stromal\ fibroblasts\ for\ staining\ intensity\ k)]$$

2. MSC conversion to TAF

Cell lines

Mesenchymal stem cells (MSC) lines were cultured from primary mesenchymal stem cells at the Center for Stem Cell Biology and Tissue Engineering of Sun Yat-Sen University. Human esophageal squamous cell carcinoma (ESCC) cell lines Eca–109 and TE–1 were purchased from the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences.

Preparation of culture supernatant of Eca–109 and TE–1 cells and induction of MSC conversion into TAF

Eca–109 and TE–1 cells were seeded separately in a 75cm^2 flask and grown in low-glucose DMEM medium without serum and without antibody for three consecutive days. The supernatant was then collected, aliquoted and stored at –80°C.

MSC cells were seeded into a 6-well plate at 2×10^5/well and cultured overnight. After complete aspiration of the growth medium, Eca–109 and TE–1 cells supernatant was added in replacement for the experimental group, while low-glucose DMEM media (with serum and double antibodies) was added in replacement for the control group. The solutions were cultured for 12–16 days with the new growth media replaced every two days. Cell RNA and proteins were collected for further analysis.

RNA extraction, reverse transcription and real-time polymerase chain reaction (PCR)

Total RNAs was isolated from supernatant of cancer cells using the tri-reagent TRIzol®, according to the manufacturer's protocol, followed by the determination of its concentration and treatment with RNase-free DNase I for complete removal of genomic DNA. Reverse transcription of total RNAs was carried out using the M-MLV reverse transcriptase according to the manufacturer's protocol. Later, the reaction mixture was incubated at 42°C for 60 min and 70°C for 10 min. After completion, cDNA was stored at –

20°C. The cDNA was then subjected to real-time quantitative PCR for the evaluation of the relative mRNA levels of β-actin, α-SMA and S100A4 with the corresponding primer pairs:

β-actin sense strand: 5'-GTGGGGCGCCCCAGGCACCA –3',

β-actin antisense strand: 5'-CTCCTTAATGTCACGCACGATTTC–3';

α-SMA sense strand: 5'-CATGTCGTCCCAGTTGGT–3',

α-SMA antisense strand: 5'-GGCTGTTTTCCCATCCATTGTG–3';

S100A4 sense strand: 5'-GAAGTCCACCTCGTTGTCCCT–3',

S100A4 antisense strand: 5'-ACCTTCCACAAGTACTCGGGCAAA–3'.

The amplification conditions were 95°C (5 min) and 45 cycles of 95°C (30 sec), 60°C (30 sec) and 72°C (30 sec).

Western blotting for Protein samples from tissues and cell cultures

For total protein extraction, the cells were floated in ice-cold lysing buffer, harvested by scraping and fragmented by ultrasonic waves three times in 4 seconds at 4℃. After a short centrifugation and boiling at 100℃ for 5 min, the extraction mixture was then centrifuged (12000rpm at 4℃ for 3 min), aliquoted and stored at –80°C. The samples were electrophoresed on a SDS-polyacrylamide gel and electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were then washed in TBST (25ml) for 10 min and blocked with 5% skimmed milk in 1×TBST. Next, the membranes were cut into strips and incubated in anti-GAPDH overnight at 4℃ on a vibrating platform. After rinsing 3 times in TBST for 5 min each, the strips were incubated at room temperature with goat anti-mouse or anti-rabbit secondary antibodies conjugated with Horseradish Peroxidase (HRP Labelled, 1:5000) for an hour on a vibrating platform, followed by three rinses in 1×TBST for 10 min each. The signal was visualized using an enhanced chemiluminescence solution followed by exposure to X-ray films.

Statistical analyses

All statistical analysis were carried out using SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA). They included the student's t-test to compare means, the logistic regression for multivariate analysis and the Chi-square test for univariate analysis. Data were expressed as mean±SD and the level of statistical significance was defined for $P<0.05$.

Results

1. TAF expression in esophageal squamous cell cancer tissues

1.1. Population

68 cases met the inclusion criteria including 54 males and 14 females. Their clinicopathologic features association α-SMA and S100A4 expressions are respectively summarized in the table below (Table 1).

1.2. Expression of α-SMA

α-SMA expressed positively in 32 cases (47.0%), with a high expression recorded in tumor stroma, mainly within the cytoplasm of stromal fibroblasts (Figure 1 A, B). However, α-SMA was not expressed in cancer cells and normal esophageal stromal fibroblasts (Figure 1 C, D).

1.3. Expression of S100A4

S100A4 expressed positively in 29 cases (42.64), with a high expression recorded in tumor stroma, mainly within the cytoplasm of stromal fibroblasts (Figure 2 A, B). S100A4 was expressed scarcely in cancer cells, and was not expressed at all in normal esophageal stromal fibroblasts (Figure 2 C, D). S100A4 (A, B, C) and α-SMA (D, E, F) expression and distribution in successive ESCC tissue sections of the same specimen were shown in Figure 3.

1.4. Univariate and multivariate analysis of α-SMA and S100A4 expressions in stromal tissues

α-SMA and S100A4 expressions correlated with tumor differentiation and clinical TNM stage. And further analysis demonstrated that α-SMA expression in stromal tissues was an independent prognosticator for ESCC patient in univariate and multivariate analysis (Table 2 and 3).

2. MSC conversion to TAF

2.1. Induction of MSC conversion into TAF in cancer cells culture supernatant

Variation of mRNA levels during MSC conversion into TAF

Real-time-PCR analysis showed that mRNA expression of α-SMA and S100A4 were increased (figure 4) during MSC conversion into TAFs using supernatant medium of TE–1 and Eca–109 ESCC cell lines. The increase was more remarkable in TE–1 medium.

Variation of proteins levels during MSC conversion into TAF

Western-Blot analysis demonstrated that protein expressions of α-SMA and S100A4 were remarkably increased (figure 5 A and figure 5 B), and therefore confirmed MSC conversion into TAFs. The increase was also more remarkable in TE–1 medium.

Discussion

Myofibroblasts can be found in the microenvironment of various tumors and extensive fibroblast-activation could be linked to rapid disease progression in malignant disease. They have an established biological impact on tumorigenesis as matrix synthesizing or matrix degrading cells and play a critical role in cancer aggressiveness and metastasis [8] [9]. α-SMA and S100A4 expressions are recognized not only in the TAFs but also in smooth muscle cells, thus suggesting limitations for interpretation of their stromal positivity [10] [11].

In our study, we detected α-SMA and S100A4 expression in TAFs within the ESCC stroma of all 68 clinical specimens using immunohistochemistry staining, confirming the location of TAFs within the matrix of cancer cells as stated by other authors [12]. Two important findings are worth pointing out. First, α-SMA and S100A4 are mainly expressed within the cytoplasm of stromal fibroblasts, but not expressed in normal esophageal stromal fibroblasts. In addition, S100A4 was expressed scarcely in cancer cells, while α-SMA was not at all. Second, both TAFs markers were expressed in the tumor stroma in form of fibroblast-like cells. The present study therefore demonstrates that α-SMA and S100A4 expressions as surface markers of TAFs could be a useful marker for the biologic behavior of ESCC and prediction of the outcome. Their expression raised was detected by immunohistochemical analysis to various extents. However, the mechanism whereby α-SMA and S100A4 expressions in TAFs influence tumor aggressiveness is still not clear.

The existence of TAFs in esophageal squamous cell carcinoma was closely related to the differentiation level of tumor cells. According to the literature, the TAFs secrete an array of soluble factors including, among others, epidermal growth factor (EGF) and transforming growth factor (TGF) which promote paracrine cancer signaling pathways involved in proliferation, survival, angiogenesis, and metastasis of epithelial tumor [13] [14], and this phenomenon is correlated with tumor malignancy according to some published literatures [15] [16]. Similarly in our study, expression levels of α-SMA and S100A4 in ESCC cell matrixes from specimen of ESCC staged III or IV according to the TNM staging system correlated with tumor differentiation and TNM staging in univariate and multivariate statistical analysis, with higher expression linked to a poorly differentiation and poorer outcome, therefore making them important independent prognosticator for ESCC (Table 3–2, 3–3). These findings are in accordance with reports from other researchers. [15, 16, 17]

Several studies show a preponderant role of TAFs during cancer metastasis [18][19], by means of autocrine secretion of cytokines and enzymes. Lymph nodes metastasis is an important route of esophageal cancer metastasis [20, 21]. Results from our experiment showed that expression levels of α-SMA and S100A4 and lymph nodes metastasis status were not significantly related ($p>0.5$), due probably

to the small size of our sample. Nevertheless, our results suggest that the degree and localization of α-SMA and S100A4 expressions in CAFs may be some of the key element of the cancer stromal microenvironment responsible for promoting cancer invasion and metastasis in ESCC.

More importantly, results from our study also demonstrated that MSCs can be converted into TAFs using supernatant medium of TE–1 and Eca–109 ESCC cell lines, thus enhancing the transfer and migration ability of ESCC cells by promoting cells epithelial to mesenchymal transition (EMT). Reports show that bone marrow derived MSC cells are one of the source of TAFs in tumor tissue [9, 22] via a process including their differentiation into fibroblasts before being converted into TAF. Additionally, some reports have suggested that MSCs can also promote carcinoma growth and lymph node metastasis when co-injected with esophageal cancer cell lines (Eca109 and TE–1) in nude mice [23]. Findings from our experiment are in agreement with those reported.

Conclusion

Our study confirmed the existence of TAFs in esophageal carcinoma, deriving from mesenchymal stem cells. Their expression in tissues of esophageal squamous cell carcinoma was also closely related to metastasis and poor prognosis. They can be identified by their unique cell surface markers which levels of expression were closely related to the degree of malignancy and tumor TNM stage, making them potential prognosticators of ESCC proliferation, invasiveness and metastasis. However, the specific mechanism underlying their effects on cancer progression needs to be further elucidated, as they represent an important target for anticancer treatments of ESCC.

Declarations

Acknowledgments

We declare no conflicts of interest in connection with the current study.

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Availability of data and material

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Authors' contributions

Conceived and designed the experiments: WY, APX, TW, and ZC. Performed the experiments: WY and TW. Acquired data: WY, BX, HZ, JZ, and CS. Analyzed the data: WY, TW, and AC. Wrote the paper: WY, AEB, TW and ZC. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The use of human materials was approved by the Medical Ethical Committee of The First Affiliated Hospital, Sun Yat-sen University (Full name of the board/committee: the Medical Ethical Committee of The First Affiliated Hospital, Sun Yat-sen University). We confirm that written informed consent from the donor or the next of kin was obtained for use of this sample in research.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Tables

Table 1. Association of α-SMA and S100A4 with clinicopathologic features.

Characteristics		*n*	α-SMA expression *n* (%)		*P*-value*	S100A4 expression *n* (%)		*P*-value*
			Positive	Negative		Positive	Negative	
Gender	Male	54	24 (44.44)	30 (55.56)	0.396	24 (44.44)	30 (55.56)	0.696
	Female	14	8 (57.14)	6 (42.86)		5 (38.46)	8 (61.54)	
Age (y)	≤60	37	16 (43.24)	21 (56.76)	0.491	16 (44.44)	20 (55.56)	0.836
	>60	31	16 (51.61)	15 (48.39)		13 (41.94)	18 (58.06)	
Differentiation	Poor	24	7 (29.17)	17 (70.83)	0.049*	7 (29.17)	17 (70.83)	0.201
	Moderate	36	19 (52.78)	17 (47.22)		17 (48.57)	18 (51.43)	
	Well	8	6 (75)	2 (25)		5 (62.5)	3 (37.5)	
TNM stage	I	5	0 (0)	5 (100)	0.049*	1 (20)	4 (80)	0.515
	II	40	18 (45)	22 (55)		17 (42.5)	23 (57.5)	
	III+IV	23	14 (60.87)	9 (39.13)		11 (50)	11 (50)	
Lymph nodes	Positive	24	15 (62.5)	9 (37.5)	0.061	13 (56.52)	10 (43.48)	0.114
	Negative	44	17 (38.64)	27 (61.36)		16 (36.36)	28 (63.64)	

*Chi-square test

Table 2. Univariate and multivariate analysis of α-SMA expression.

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
α-SMA expression						
Positive	0.819	0.448 - 1.497	0.517	0.48	0.249 - 0.926	0.029*
Negative	1.000			1.000		
Differentiation			0.064			0.017*
Moderate	1.481	0.756 - 2.901	0.253	1.582	0.732 - 3.418	0.243
well	3.045	1.202 - 7.716	0.019*	4.502	1.58 - 12.831	0.005*
TNM classification			0.03*			0.287
II	4.546	0.615 - 33.591	0.138	3.65	0.439 - 30.331	0.231
III + IV	8.45	1.123 - 63.595	0.038*	9.728	0.574 - 164.792	0.115
Sex						
Male	1.01	0.486 - 2.1	0.979	0.881	0.395 - 1.964	0.757
Female	1.000			1.000		
Age						
≥60 years	0.894	0.494 - 1.616	0.71	0.966	0.523 - 1.784	0.912
<60 years						
Lymph nodes metastasis						
With	2.038	1.12 - 3.707	0.02*	1.08	0.152 – 7.682	0.939
Without	1.000			1.000		

*Chi-square test

Table 3. Univariate and multivariate analysis of S100A4 expression.

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
S100A4						
Positive	1.074	0.587 – 1.962	0.818	0.783	0.413 – 1.485	0.453
Negative	1.000			1.000		
Differentiation			0.064			0.038*
Moderate	1.481	0.756 – 2.901	0.253	1.323	0.618 – 2.832	0.471
well	3.045	1.202 – 7.716	0.019*	3.73	1.314 - 10.589	0.013*
TNM classification			0.03*			0.477
II	4.546	0.615 - 33.591	0.138	3.265	0.392 - 27.172	0.274
III + IV	8.45	1.123 - 63.595	0.038*	5.215	0.306 – 88.979	0.254
Sex						
Male	1.01	0.486 – 2.1	0.979	0.948	0.409 – 2.196	0.901
Female	1.000			1.000		
Age						
≥60 years	0.894	0.494 – 1.616	0.71	0.989	0.531 – 1.841	0.972
<60 years	1.000			1.000		
Lymph nodes metastasis						
With	2.038	1.12 – 3.707	0.02*	1.452	0.19 - 11.081	0.719
Without	1.000			1.000		

HR, hazard ratio; LN, lymph nodes. *Chi-square test

Figures

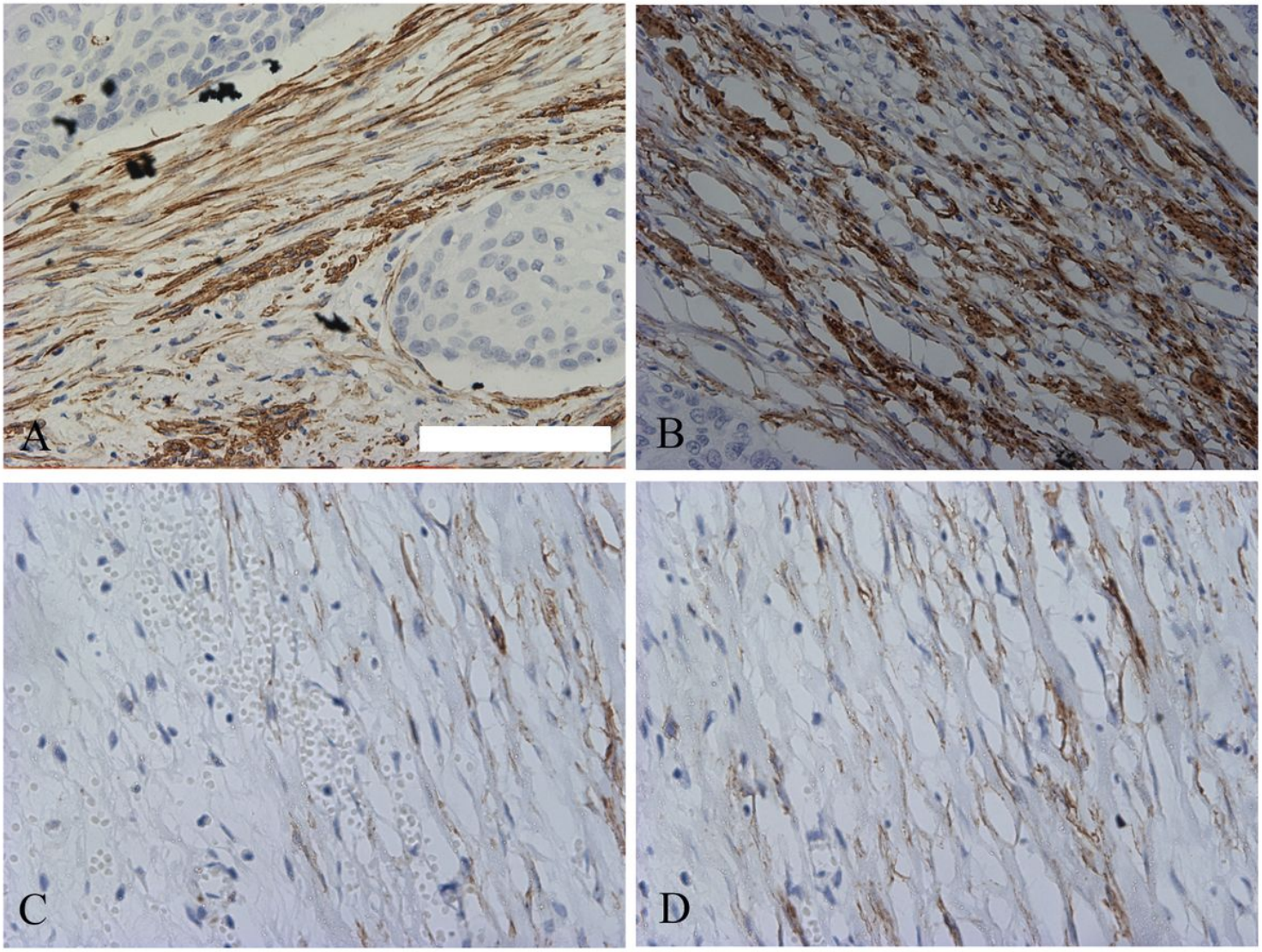

Figure 1

α-SMA expression within the cytoplasm of stromal fibroblasts on immunohistochemically stained ESCC tissue sections (positive: A, B; negative: C, D); Scale bars: 100 μm.

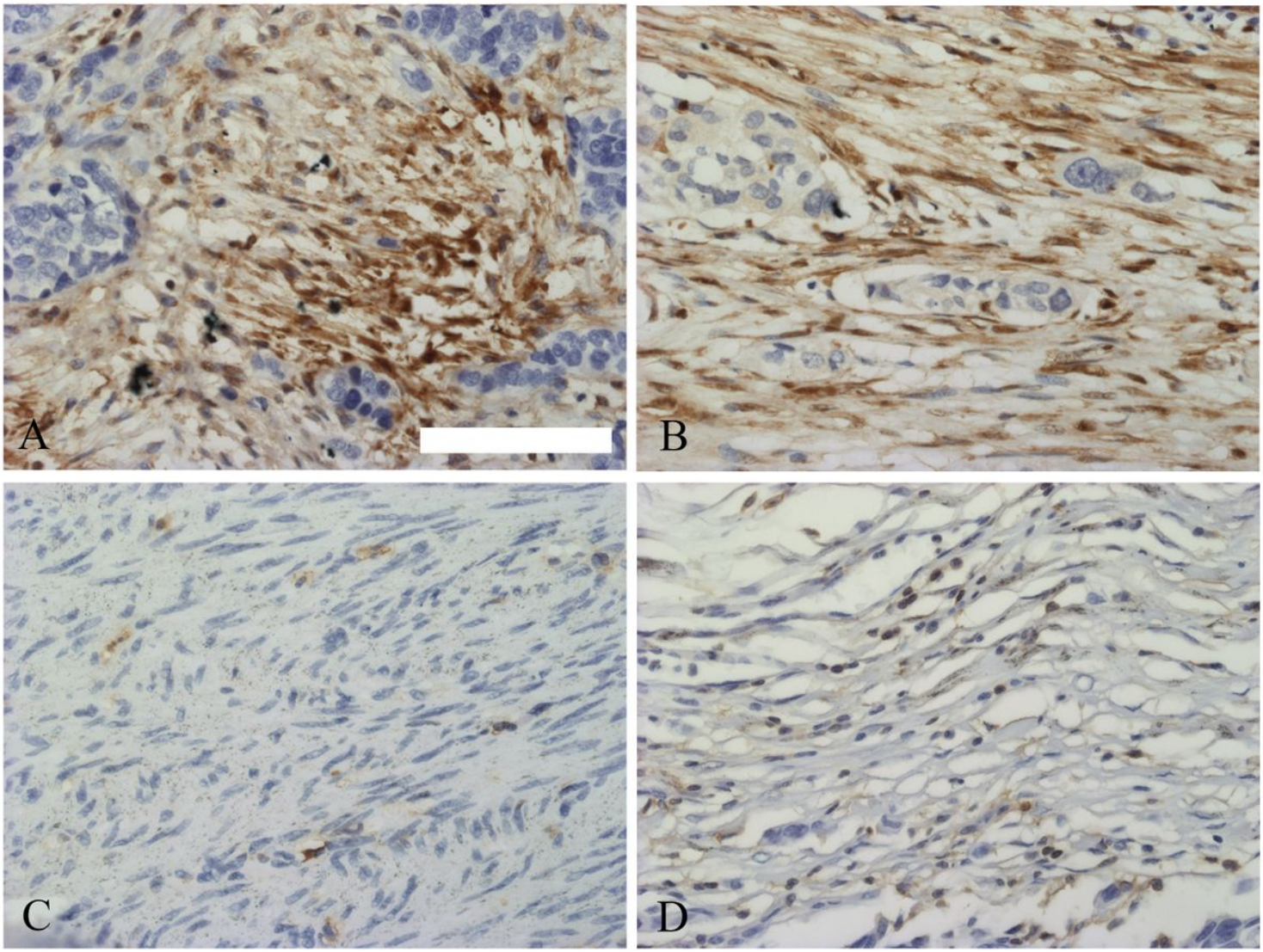

Figure 2

S100A4 expression within the cytoplasm of stromal fibroblasts on immunohistochemically stained ESCC tissue sections (positive: A, B; negative C, D); Scale bars: 100 μm.

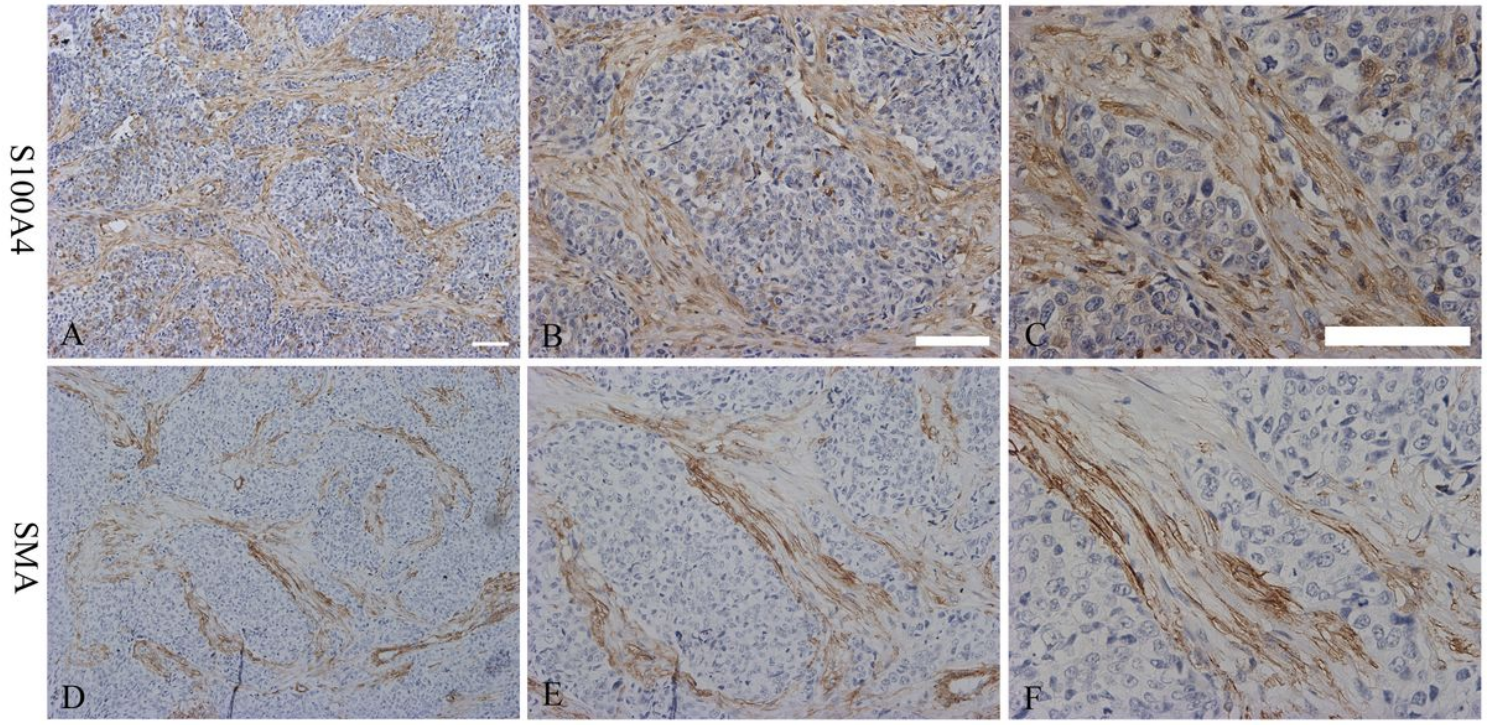

Figure 3

S100A4 (A, B, C) and α-SMA (D, E, F) expression and distribution in successive ESCC tissue sections of the same specimen. Scale bars: 100 μm.

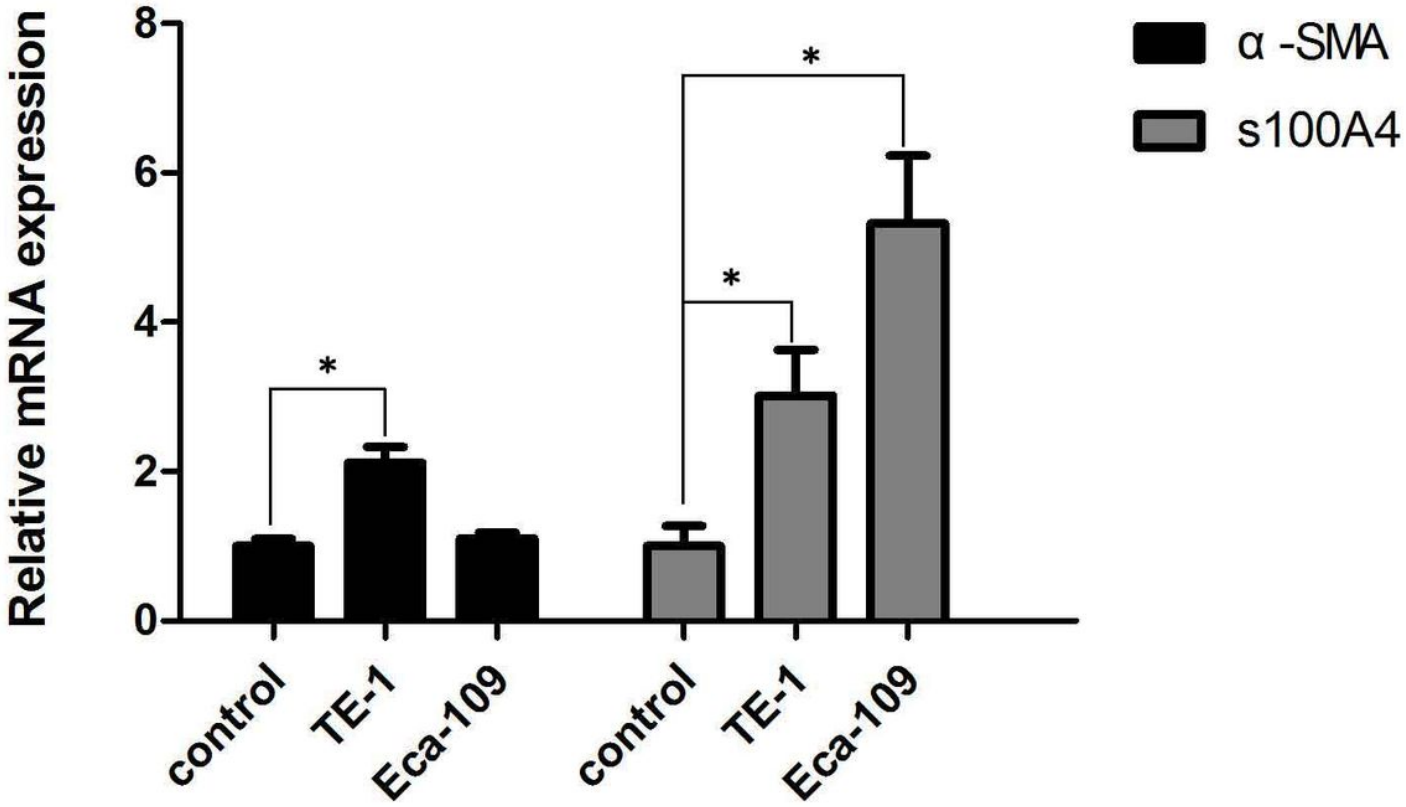

Figure 4

mRNA expression of α-SMA and S100A4.

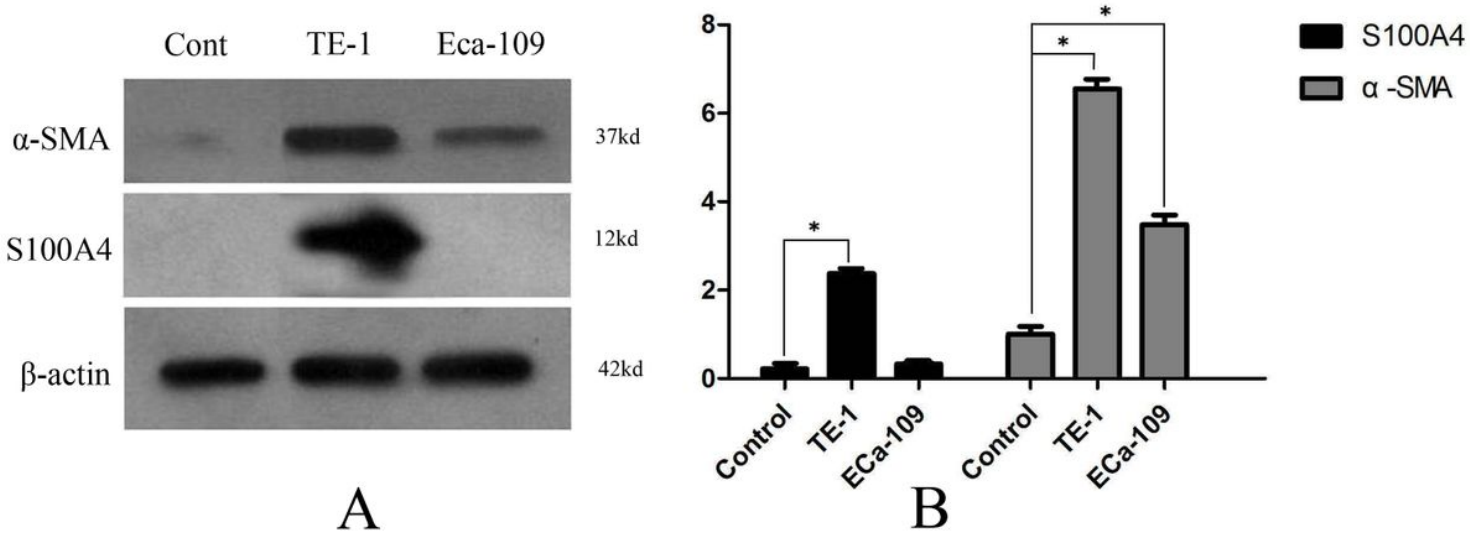

Figure 5

(A, B). Protein expression of α-SMA, S100A4 and β-actin.