

Tumor-Associated Fibroblasts and Microvessels Contribute to the Expression of Immunosuppressive Factor Indoleamine 2, 3-Dioxygenase in Human Esophageal Cancers

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Abstract Recent studies have provided considerable evidence to support the hypothesis that tumor stroma plays a crucial role in the induction of immune tolerance to human cancers. Here, we investigated the contribution of reactive stromal tumor-associated fibroblasts (TAFs) and microvessels to the immunosuppressive factor indoleamine 2,3-dioxygenase (IDO) expression in the ESCC microenvironment. The immunohistochemical (IHC) analyses demonstrated a significant increased densities of TAFs and microvessels in the ESCC stroma, double IHCs showed that these increased TAFs and microvessels were with a high proliferation activity. Further IHC examinations revealed that increased expression of IDO were frequently observed in the stromal cells with TAF morphology and microvessels. Double immunofluorescence examinations confirmed the colocalization of IDO positive cells with SMA-alpha positive TAFs and CD34 positive endothelial cells in the ESCC stroma. Our current findings strongly suggest that the activated stromal TAFs and endothelial cells of microvessels contribute to the expression of IDO and then the orchestration of immunosuppressive microenvironment.

Keywords Fibroblasts · Endothelial cells · Indoleamine 2,3-dioxygenase · Immunosuppression; carcinoma; esophagus

Abbreviations

| | |
|------|------------------------------------|
| ESCC | Esophageal squamous cell carcinoma |
| IHC | Immunohistochemistry |
| IDO | Indoleamine 2,3-dioxygenase |
| MVD | Microvessel density |
| TAF | Tumor-associated fibroblast |

Introduction

Esophageal cancer including squamous cell carcinoma (ESCC) and adenocarcinoma is a serious healthcare problem worldwide because of its increasing morbidity and high mortality [1]. ESCC presents as a great majority in some Asian countries such as China [1], our location (Henan Province, North China) has been reported to have the highest incidence of esophageal cancer in the world and over 90% cases are ESCCs [2]. During the last three decades, the long-term survival rate for patients with ESCC has not improved substantially, and the prognosis of ESCC remains among the worst sites for all cancers with respect to survival. Therefore, to improve clinical outcome, attempts to better understand the controlling of ESCC growth and progression have been made; results showed that tumor immune escape, which is highly associated with tumor antigen-specific immune tolerance as well as with the tumor immunosuppressive microenvironment, might play a role in the progression of human ESCC. However, the precise mechanisms contribute to the progression of ESCC are not fully understood.

It has being recognized that host immunity at the primary site is one of the potential factors influencing the progression and prognosis of human cancers [6]. However, tumor cells can use diverse strategies to inhibit the local specific-tumor immunity in order to escape attack and prolong their survival, then progress persistently. Recent progress in cancer biology has suggested that human cancers can shape a tolerogenic

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environment enabling cancer cells to avoid immune evasion by releasing immunosuppressive factors that derived from both tumor cells and non-tumor cells [3–5]. Indoleamine 2,3-dioxygenase (IDO), an enzyme that degrades the essential amino acid l-tryptophan along the kynurenine pathway, has attracted considerable attention as a novel immunosuppressive factor in various types of human cancers [6–18]. Increased synthesis of IDO protein may suppress host anti-tumor immunity and then contribute to cancer cell escape from attack by the host immune system [16, 23–25]. In the tumor microenvironment, expression of IDO by cancer cells as well as by stromal cells have been detected [19]. Increased activity of IDO can significantly inhibit cancer cell proliferation and induces apoptosis in T cells and natural killer cells through tryptophan depletion and production of toxic tryptophan catabolites [10, 11]. In addition, IDO can activate the immunosuppressive activity of regulatory T-cell (Tregs), which also contributes to the promotion of immune tolerance in the tumor microenvironment [12]. The use of IDO inhibitors as single agent or in combination with other treatment modalities in human cancers have shown to enhance dendritic cell immunogenicity and lytic ability of tumor antigen-specific T cells [20], which makes it to an interesting target for immunotherapy [17, 19].

In patients with ESCCs, Sakurai et al. have reported increased expression of IDO at mRNA level expression in both the tumor tissue and the blood of ESCCs [20]. Our group has previously reported a significant increased expression of IDO in ESCC tissues from a high incidence area in China, which is mainly expressed by the ESCC cells and the stromal cells [14]. More recently, two other Chinese groups have further shown that the expression of IDO may impair the functions of infiltrating CD8 positive T lymphocytes [21] and was correlated with poor clinical outcome in patients with ESCC [15]. These results confirmed our previous findings and supported the hypothesis that IDO is an important immunosuppressive mediator and influences the clinical outcome of ESCC.

According to the recent data, production of IDO by both the ESCC cells and stroma cells has been described [7, 14, 22]. Tumor associated fibroblast (TAF) is a subpopulation of stromal fibroblasts that is the major cellular component of tumor stroma, and has been hypothesized to drive tumor progression through the deposition of extracellular matrix (ECM) proteins, the secretion of growth factors and the producing of immunosuppressive mediators [19]. Recent progression in tumor stroma studies also suggested that TAFs

could produce high amount of immunoregulatory factors and contribute to the formation of inflammatory microenvironment. [19–22]. In addition, new vascular formation (angiogenesis) is another histological feature for tumor stroma. It has generally recognized the importance of new vascular formation for the tumor growth, invasion and metastasis. Since the activated TAF and angiogenic response in the ESCC has been reported by our and other groups [23, 24], a role of stromal cells in contributing to the expression of IDO in the tumor microenvironment cannot be excluded [9, 14, 25]. We, therefore, design this study to examine our hypothesis that reactive TAFs and endothelial cells of microvessels might contribute to the orchestration of immunosuppressive microenvironment by releasing of IDO in patients with ESCC.

Materials and Methods

Patients

The research was conducted on a group of 50 curative surgically resected tumor tissue paraffin blocks (male/female ratio: 31/19; age ranging from 44 to 75 years) from ESCC patients admitted to the Second Affiliated Hospital of Zhengzhou University between January 2012 and December 2015. Ten adjacent non-tumor esophageal tissues were served as controls (male/female ratio: 9/1; age ranging from 52 to 70 years). No one received radiotherapy and/or chemotherapy preoperation. Representative sections were cut at a thickness of four μm and then stained with hematoxylin-eosin (*H&E*) for routine histological diagnosis. Basic information of ESSC is summarized in Table 1. This work was approved and supported by Local Medical Research Committee of the Second Affiliated Hospital of Zhengzhou University.

Immunohistochemical Examination of TAFs, Microvessels and IDO in ESCC Tumor Stroma

Immunohistochemistry (IHC) for the examinations of TAFs, microvessels and IDO positive cells in ESCC tumor stroma was performed in 4 μm sections with Vectastain *Elite* Universal ABC-HRP kits (Vector Lab., Barrington, CA, USA) according to the manufacturer's instructions and our published methods [26, 27]. Antigen retrieval for IHCs with SMA-alpha (to label TAFs) and CD34 (to label

Table 1 Basic information of ESSC patients

| | N | TNM | | | Differentiation | | | Lymph node involvement | |
|------|----|-----|----|-----|-----------------|------------|-----|------------------------|----|
| | | I | II | III | Well | Moderately | Low | + | - |
| ESSC | 50 | 6 | 35 | 9 | 8 | 37 | 5 | 15 | 35 |

endothelial cells of microvessels) antibodies was achieved by boiling sections in 0.01 M sodium citrate buffer (pH 6.0) through microwave processing for 15 min, antigen retrieval for IDO IHC was achieved by incubating the sections with ready-to-use Proteinase K solution (Dako Cor, Carpinteria, CA, USA) for 10 min respectively. The following primary antibodies were used: Mouse anti-human smooth muscle actin (SMA)-alpha monoclonal antibody (Clone 1A4, Dako Cor., Carpinteria, CA, USA), mouse anti-CD34 monoclonal antibody (Dako Cor., Carpinteria, CA, USA), and mouse anti-human IDO monoclonal antibody (Oriental Yeast Co., LTD., Tokyo, Japan) over night at 4 °C. Then, the sections were incubated with the 3-amino-9-ethylcarbazole (AEC; Vector Laboratories, Burlingame, CA, USA) was used as chromogen and Mayer's hematoxylin as the counterstained. The negative control slides were made: (1) primary antibodies were substituted with the isotype-matched control antibodies; (2) secondary antibodies were substituted with phosphate buffered saline.

Double IHCs to Define Proliferation Activity in Stromal TAFs and Microvessels

To define the proliferation activity of TAFs and microvessels in the ESCC microenvironment, double IHCs were performed using EnVision G72 Doublestain System kit (Dako Demark, Glostrup, Denmark) with antibodies PCNA (rabbit polyclonal, Abcam, Cambridge, UK)/SMA-alpha, PCNA/CD34 according to the manufacturer's instructions and the method as previously described in our publication [14, 28, 29]. The visualization for IDO- and PCNA-immunoreactivities was detected with the enzyme substrate 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Vector Lab., Barrington, CA, USA) and the visualization for SMA-alpha- and CD34-immunoreactivities were with *Permanent Red* substrate solution (Dako Demark, Glostrup, Denmark). Slight nuclear counterstaining with Mayer's hematoxylin was applied.

Double Immunofluorescence (IF) Staining to Define the Coexpression of IDO with TAFs and Microvessels in the ESCC Stroma

To define the coexpression of IDO with TAFs and microvessels in the ESCC stroma, double IF staining with IDO (rabbit polyclonal, Santa Cruz Biotechnology, Inc. USA)/SMA-alpha, IDO/CD34 antibodies were performed according to our previous published methods [30, 31]. In brief, following both IDO/SMA-alpha or IDO/CD34 antibodies incubation at 4 °C overnight, slides were developed with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Jackson ImmunoResearch Lab., West Grove, PA, USA; to visualize IDO immunoreactivity). Then, with

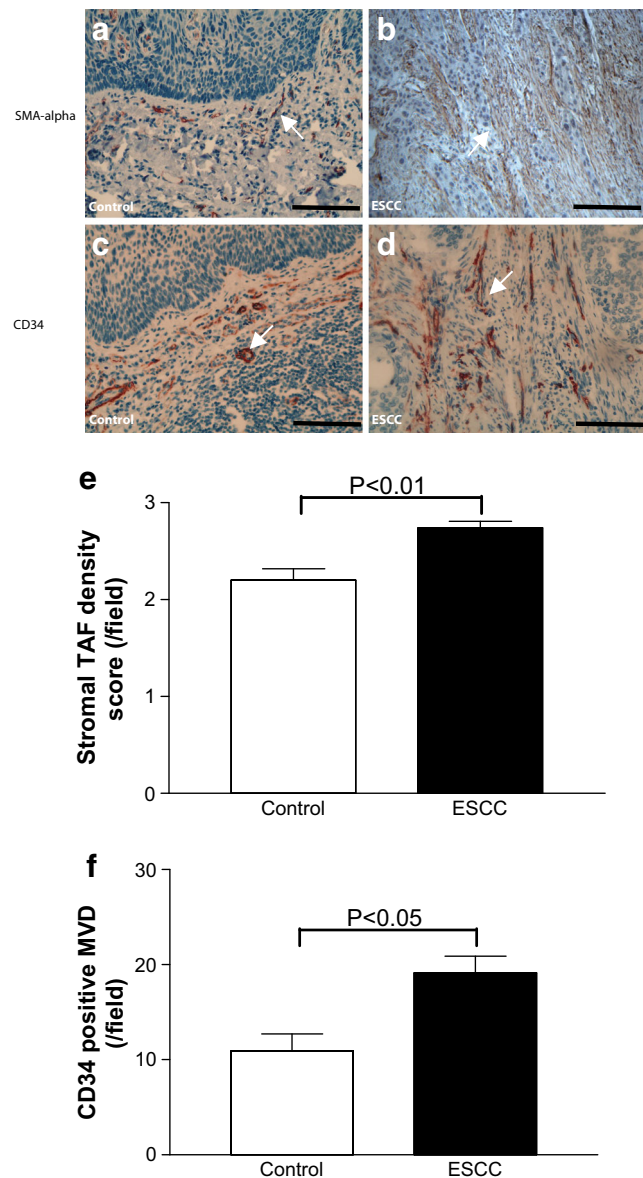


Fig. 1 Photograph and graphic presentations of reactive TAFs and endothelial cell formation in the ESCC tumor stroma. Immunohistochemical (IHC) results show that increase in TAFs positive for SMA-alpha were observed in the ESCC stroma (Fig. 1b) in relative to the control stroma (Fig. 1a). The formation of CD34 positive endothelial cell was also increased in the ESCC stroma (Fig. 1d) in relative to the control (Fig. 1c). Graphic analysis of semi-quantification results confirmed above IHC observations and demonstrated an increase of TAFs (Fig. 1e) and microvessels density (MVD) (Fig. 1f) in the ESCC stroma. (A-D: single IHC, original magnification 200×)

incubation of tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary IgG antibody (Jackson ImmunoResearch Lab., West Grove, PA, USA; to visualize SMA-alpha or CD34 immunoreactivity) for 30 min and sealed with fluorescence cover medium (Dako, Carpinteria, CA, USA) for the observation by confocal microscopy (LSM-510 meta, Carl Zeiss, Jena, Germany).

Morphometric Evaluation

All stained slides were examined under light microscopy. The density of SMA positive TAFs in the ESCC stroma was evaluated according to the methods described in our previous publication [23, 28]. The density of CD34 positive microvessels (MVD) and IDO positive cells in the tumor stroma was absolutely quantitated according to our published method: briefly, the microvessels with CD34 immunoreactivity and IDO positive cells located in the ESCC stroma was counted in at least 5 optical high-power magnification fields ($\times 400$) with abundant distribution from each slide [11, 16]. The average values per slide were used for statistical analysis.

Statistical Analysis

The results were expressed as the mean \pm SEM (standard error of the mean) unless otherwise stated. Statistical significance was evaluated by the Mann–Whitney test. Value of $P < 0.05$ was considered significant.

Results

Increased Densities of Reactive TAFs and Microvessels Were Observed in the ESCC Stroma

TAFs are the major cellular components of cancer stroma that activated in response to the ESCC carcinogenesis. In this study, we used SMA-alpha antibody to define TAFs in the ESCC tumor stroma.

The results showed that SMA-alpha positive TAFs could be observed in the tumor stroma in all the cases of ESCC. Increased SMA-alpha positive TAFs were diffusely located in the whole tumor stroma but were particularly dense in the stromal region between tumor masses (Fig. 1b) as compared

with the controls (Fig. 1a). When the density of SMA-alpha positive TAFs in the tumor stroma was graded, it showed that the grading scores were greatly increased (Fig. 1e, *black bar*) relative to the controls (Fig. 1e, *white bar*).

Enhanced angiogenesis is one of the main reactive response in the tumor stroma and plays the important role in supporting ESCC growth and progression. In this study, endothelial cell formation (angiogenesis) in the ESCC stroma was evaluated by the analysis of total microvessel density (MVD) labelled with CD34 immunoreactivity.

The results of CD34 IHC analysis showed that a high density of MVD was observed in the ESCC stroma (arrow in Fig. 1d) relative to the controls (Fig. 1c); the counting data confirmed that MVD in the ESCC stroma was significantly increased as compared with that in controls (Fig. 1f, *black bar*).

Double IHCs Revealed that TAFs and Microvessels in the ESCC Stroma Were with a High Rate of Proliferation

The proliferation activity of reactive TAFs and microvessels in the ESCC stroma was evaluated with double IHCs. The results showed that the proliferation activity labelled by PCNA immunoreactivity was frequently observed in SMA-alpha positive TAFs (Fig. 2a) and CD34 positive endothelial cells of microvessels (Fig. 2b).

Increased IDO Expression in TAFs and Microvessels in the ESCC Stroma

IDO immunoreactivity can be observed in both ESCC cells and stromal cells. Here we only describe its presentation in TAFs and microvessels in the ESCC stroma.

Increased expression of IDO was observed in the ESCC stroma relative to the controls. From a morphological view, many IDO positive cells present in the ESCC stroma were

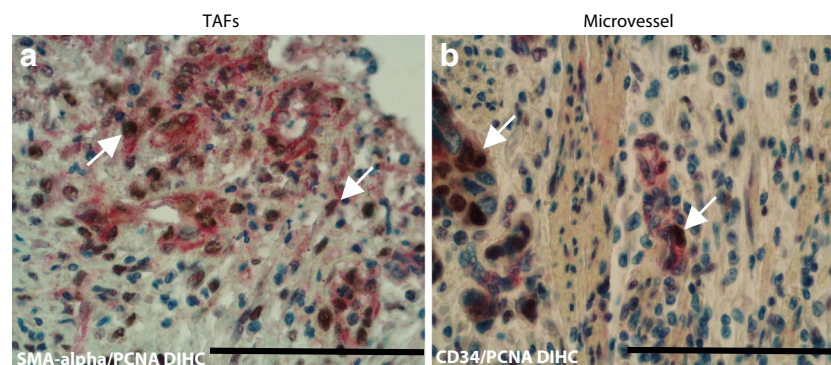


Fig. 2 Double IHCs demonstrated that TAFs and endothelial cells of microvessels in the ESCC stroma were with a high rate of proliferation. Double IHCs with SMA-alpha/PCNA and CD34/PCNA antibodies revealed a high activity of proliferation located in the TAFs (Fig. 2a,

Red color for SMA-alpha and *Brown color* for PCNA) and endothelial cells of microvessels (Fig. 2b, *Red color* for CD34 and *Brown color* for PCNA) in the ESCC stroma. (Fig. 2a, b, double IHC, counterstained with hematoxylin, original magnification 400 \times)

likely TAFs (Fig. 3a and enlarged image 3B) and microvessels (Fig. 3c and enlarged image 3D). The counting data confirmed significant increased densities of IDO positive TAFs (Fig. 3e, *black bar*) and IDO positive MVD (Fig. 3f, *black bar*) in the ESCC stroma.

Double IF Staining Showed a Coexpression of IDO with TAFs and Microvessels in the ESCC Stroma

Then we further examined the expression of IDO in TAFs and endothelial cells of microvessels in the ESCC stroma with double IF staining. The results revealed that IDO immunoreactivity was frequently coexpressed with SMA-alpha positive TAFs (Fig. 4a–c) and CD34 positive endothelial cells of microvessels (Fig. 4d–f) in the ESCC stroma.

Discussion

The current study has demonstrated that reactive stromal TAF and endothelial cell of microvessel expressed IDO in the ESCC. These findings suggest that ESCC stromal cells contribute to the formation of immunosuppressive microenvironment.

Strong stromal response is a key feature of the tumor microenvironment [23, 28]. According to the recent evidence, tumor stroma may act as a double edge sword in response to carcinogenesis: to limit transformed cells when the reaction is in a prompt and efficient manner; alternately, facilitate the carcinogenesis under certain conditions such as chronic inflammation via releasing a great number of growth factors, cytokines and immunosuppressive mediators to participate in the epithelial-stroma interaction in many types of human cancers [32, 33]. In this study, we were able to demonstrate a significant increased density of TAFs that was with a high proliferation activity. This finding suggests that fibroblasts in the ESCC microenvironment are greatly activated in response to the esophageal carcinogenesis, and provide a histological fundament for TAFs in producing of immunoregulatory factors. Indeed, we have demonstrated that TAFs in the ESCC stroma were highly positive for the immunoreactivity of IDO, and double IFs with IDO/SMA-alpha antibodies further confirmed that IDO immunoreactivity is frequently colocalized with SMA-alpha immunoreactivity.

Enhanced angiogenesis has been reported to participate the tumor growth, invasion and metastasis in ESCC. We have previously reported that the expression of proangiogenic factors vascular endothelial growth factor (VEGF) and interleukin (IL)-8 are significantly increased in the ESCC stroma [23]. In this study, we further examined the angiogenesis by characterized total microvessel density (MVD) in the ESCC stroma. Our results demonstrated that CD34 positive MVD was greatly increased, and proliferation rate in microvessels was increased. These results suggested that

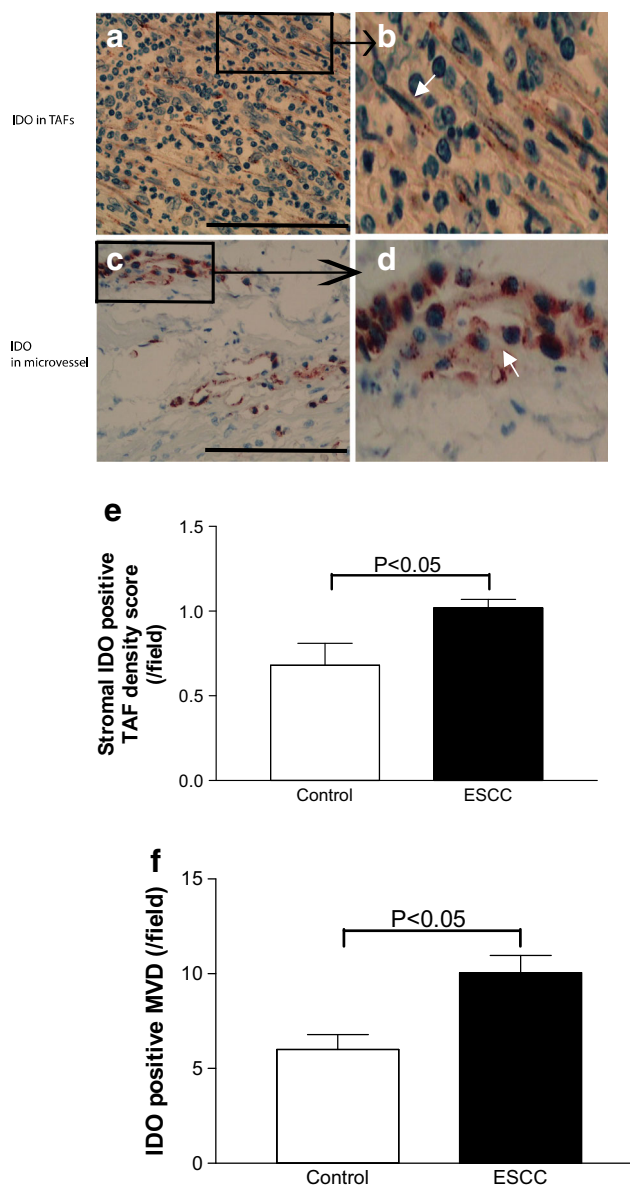


Fig. 3 The expression patterns of IDO in the ESCC stroma was evaluated with immunohistochemistry (IHC). In the ESCC stroma, the IDO immunoreactivity was frequently observed to be located in the stromal cells with TAF morphology (Fig. 3a and enlarged image 3B) and microvessels (Fig. 3c and enlarged image 3D). Graphic analysis of semi-quantification results confirmed that IDO positive TAFs (Fig. 3e) and microvessel density (MVD) (Fig. 3f) were greatly increased in the ESCC tumor stroma. (Fig. 3a, c, single IHC, counterstained with hematoxylin, original magnification 400×)

esophageal carcinogenesis results in a significant increased new vascular endothelial cell formation in the ESCC stroma. Interestingly, microvessels have also been reported the main cellular source for IDO production in other types of human cancers [7, 34–36].

There is compelling evidence that immunosuppressive mediators derived from both the tumor cells and other types of cells in the microenvironment play a major role in the extending of immune escape and facilitating of cancer progression

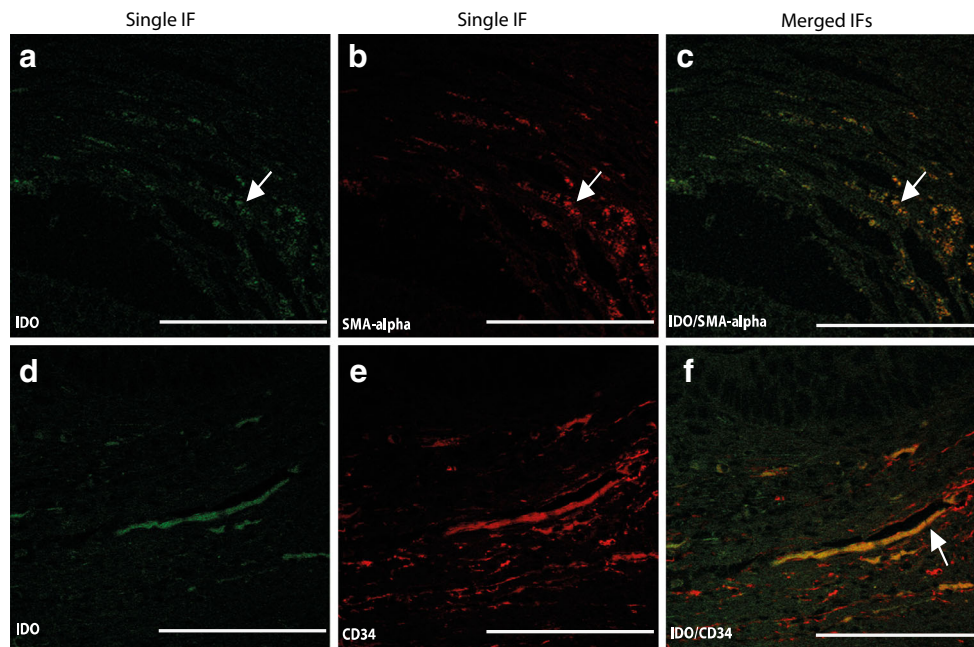


Fig. 4 a-f Double immunofluorescence staining with IDO/SMA-alpha and IDO/CD34 antibodies. Double immunofluorescence staining with IDO/SMA-alpha and IDO/CD34 antibodies confirmed that immunoreactivity of IDO (Fig. 4a, c, labelled with FITC, Green color) was frequently colocalized with SMA-alpha positive TAFs (Fig. 4b,

labelled with TRITC, Red color) and merged image Fig. 4c) and CD34 positive endothelial cells of microvessels (Fig. 4e, labelled with TRITC, Red color; and merged image Fig. 4f) in the ESCC stroma. (Fig. 4a-f, double immunofluorescence staining; Original magnification 400 \times)

[5]. IDO is one of the new known immunosuppressive mediators. A growing body evidence have showed that increased synthesis of IDO protein in human cancers suppresses host anti-tumor immunity, induces immune tolerance and then enhances cancer cell escape from host immune attack [37]. Regarding the expression of IDO in the human ESCC, several groups including ours [14, 15, 21, 37, 38] have reported that the expression of IDO is remarkably increased in the tumor microenvironment. We have previously showed that IDO is expressed by both ESCC cells and stromal cells [14]. Increased IDO in the ESCC can suppress the anti-tumor function of CD8 T cell [21] and associated with a worse prognosis [15]. In the present study, we have further confirmed previous findings that increased expression of IDO in the ESCC tumor stroma was mostly located in the stromal cells with TAF morphology and microvessels. By using single IHC and double IFs, we were able to confirm that TAFs and vascular endothelial cells of microvessels were the main cellular forms to express IDO in the ESCC stroma. Which strongly suggest that reactive stromal cells and endothelial cells of microvessels contribute to the formation of an immunosuppressive microenvironment via releasing IDO in the ESCC stroma.

In summary, we demonstrated in the current study that reactive TAFs and microvessels are essential for the expression of IDO in the ESCC, which might be one of the mechanisms for ESCC cells in inhibiting the host antitumor immunity and then promoting ESCC growth and progression. Our findings, together with the results of other studies, strongly

suggest that anti-cancer therapeutic strategies targeting this enzyme in ESCCs is needed to be investigated in the future.

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Compliance with Ethical Standards

Conflict of Interest We declare that we have no conflict of interest.

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