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ORIGINAL ARTICLE

Localization of indoleamine 2,3-dioxygenase in human esophageal squamous cell carcinomas

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Abstract Immunosuppressive factors derived from the tumor and nontumor cells present in the tumor microenvironment contribute to tumor escape from host immune attack. Recently, the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) derived from both the tumor cells and surrounding nontumor cells was found to function as a critical immunosuppressive factor. While the expression of IDO is intensively under investigation in many types of cancers, little information is available in esophageal squamous cell carcinomas (ESCC) thus far. In this study, we have therefore investigated the cellular localization of IDO in 45 ESSCs and ten morphologically normal esophageal tissues; the correlation of IDO with clinicopathological parameters was also analyzed. Immunohistochemistry (IHC) analysis revealed that the density of IDO-positive cells was increased in ESCCs relative to controls ($P<0.01$). These cells were distributed as clusters and formed a patchy pattern in both the cancerous epithelium and the surrounding noncancerous cells. Double IHC further confirmed that many IDO-positive cells in the tumor stroma were smooth-muscle-actin-alpha-positive myofibroblasts, CD68-positive macrophages, and S100-positive dendritic cells. Statistical analysis showed that the densities of IDO-positive cells were not significantly correlated with tumor clinical parameters (tumor invasion depth, node metastasis, and TNM stages) and lymphocytic infiltration. Our current findings suggested that the increased IDO expression in ESCCs is from a mixed cellular source (both cancer cells and noncancerous cells). Further studies on immune cell functional analysis are required in the future.

Keywords Immunosuppression · Carcinoma · Esophagus

Abbreviations

IDO	Indoleamine 2,3-dioxygenase
IHC	Immunohistochemistry
ESCC	Esophageal squamous cell carcinoma

Introduction

Esophageal cancer is a highly aggressive lethal malignancy worldwide with regional variations in incidence [1–3]. Asian countries have a high incidence, and the predominant histological type of esophageal cancers is esophageal squamous cell carcinoma (ESCC), which is in contrast with the reports from Western counties that a high incidence of esophageal adenocarcinoma was found. Our location (Henan Province, North China) has been reported to have the highest incidence of esophageal cancer in the world [2], and ESCC accounts for over 90% cases. In our

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Table 1 Basic clinicopathological information of ESCC patients

	Number	Tumor invasion			Lymph node involvement		Metastasis		TNM stages		
		T1	T2	T3	N0	N1	M0	M1	I	II	III
ESCC	45	1	9	35	35	10	45	0	1	34	10

province, over the past 40 years, there has been a strong attempt to make an early diagnosis for patients with ESCC, since an improved prognosis is heavily dependent on the disease stage. Unfortunately, the metastasis is often observed at the time of diagnosis, and curative surgery becomes impossible in many patients with ESCC. The prognosis is still very poor.

To seek potential therapeutic strategies for these ESCC patients with metastasis, a better understanding of the mechanisms of tumor cell growth control and progression is necessary. Several lines of evidence suggest that tumor cells can grow by escaping from the host immune system control and the malignant potential is significantly influenced by host immunity. One of the tumor's abilities to escape immune attack is to inhibit antitumor immunity by producing immunosuppressive factors [4, 5]. Indeed, a growing body of evidence has suggested that a variety of immunosuppressive factors derived from the tumor cells and surrounding cells contribute to the establishment of regional immunosuppressive networks in patients with ESCC [6–9]. Recently, indoleamine 2,3-dioxygenase (IDO), an intracellular enzyme that catalyzes the initial and rate-limiting steps in the metabolism of the essential amino acid tryptophan along the kynurenine pathway, is found to be increased and functions as a critical immunosuppressive factor in many types of human cancers [10–18]. However, little information is available concerning the expression of IDO in ESCC. The first study concerning IDO expression in patients with ESCC was reported by Sakurai et al. [19]. They showed that the mRNA level of IDO was remarkably increased in either the cancerous tissue or blood as compared with the controls. Furthermore, their statistical analysis suggested that the ESCC patients with higher levels of IDO expression had a worse survival rate than the IDO expression group with lower levels. It has been demonstrated that a variety of cells in the tumor microenvironment contribute to the IDO expression; IDO immunoreactivity is identified in both the cancer cells and the surrounding noncancerous cells (i.e., macrophages and dendritic cells) in many types of cancers [12, 20–22]. However, the phenotypic analysis of IDO expression in ESCCs is still lacking. In this study, we therefore investigated the cellular expression pattern of IDO in the tumor microenvironment and analyzed whether the IDO-positive cell density influenced the clinicopathological features in patients with ESCCs.

Materials and methods

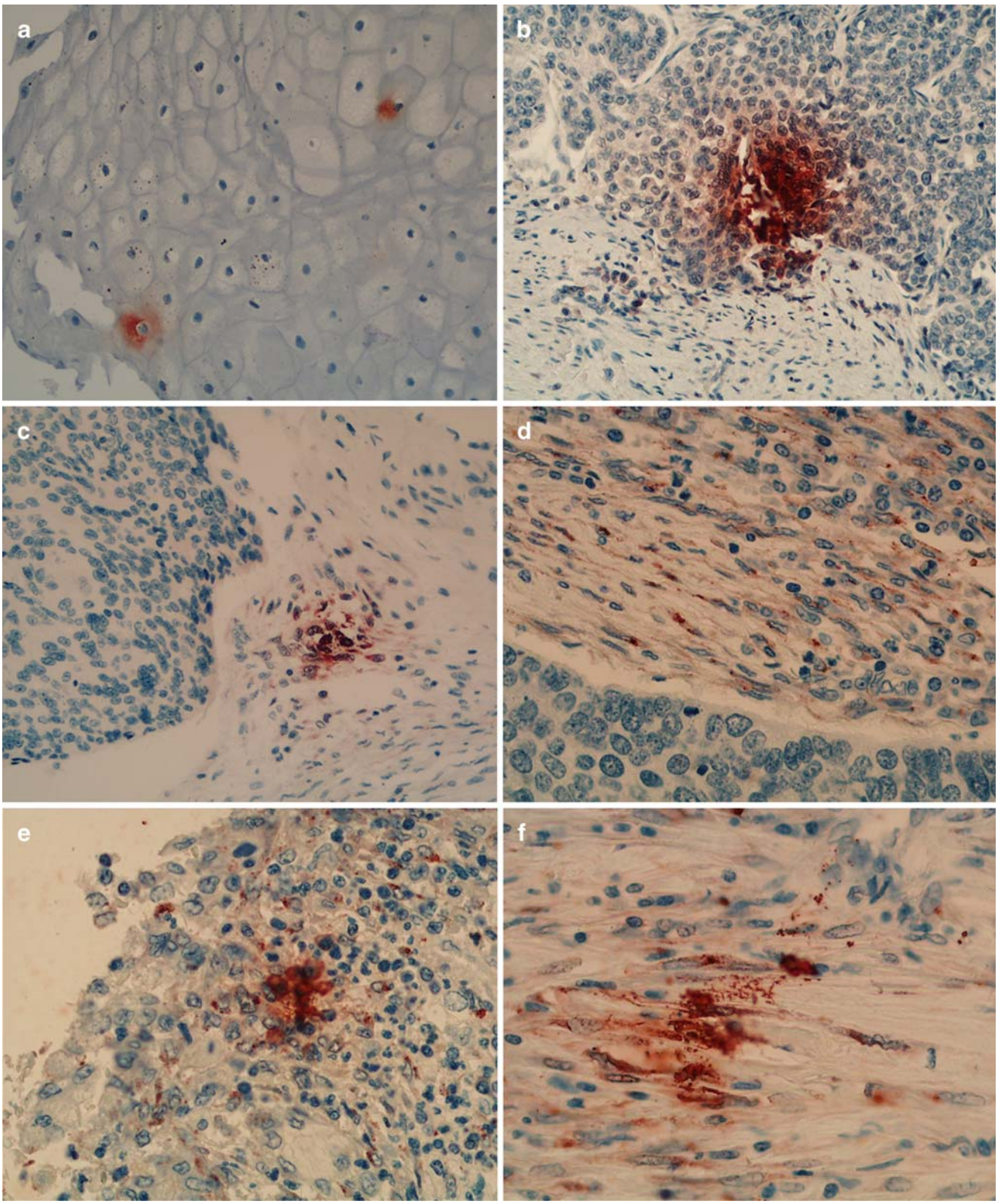
Tissue specimens from ESCCS and controls

Forty-five surgical ESCC paraffin blocks tracked from the tissue bank in the Departments of Pathology, the Fourth Affiliated Hospital of Zhengzhou University, between August 2003 and November 2008 were included in the study according to the guidelines of the protocols approved by the institutional review board. There were 28 men and 17 women ranging in age from 32 to 76 years. The tumor location was in the upper third in seven patients, in the middle third in 25 patients, and in the lower third in 13 patients (for other information, see Table 1). None of the ESCC patients received immunomodulatory therapy, chemotherapy, or radiotherapy before surgery. Ten morphological normal esophageal mucosa without pathological evidence taken by endoscopy examinations served as controls (male/female 7/3; age 26–76 years; three were taken from upper third, four from middle third, and three from lower third esophagus).

Immunohistochemical examination of IDO and tumor-associated lymphocyte expression in the tumor microenvironment

Immunohistochemistry (IHC) was performed in 4-μm sections with Vectastatin Elite Universal ABC-HRP kits (Vector Lab., Burlingame, CA, USA) according to the manufacturer's instructions and our published methods [23, 24]. Antigen retrieval was achieved by incubating the

Fig. 1 Examination of the IDO expression patterns in the tissues of ESCCs by single IHC and DIHC. IDO-positive cells were only observed in low density in the control mucosa **a**), but they were present as cluster and formed a patchy distribution pattern with a high density in ESCC epithelium **b**) and surrounding noncancerous cells **c**). In tumor stroma, many of them had fibroblast morphological features (**d**), leukocytes (**e**), and microvessels (**f**). DIHC further confirmed that those IDO-positive surrounding noncancerous cells (*brown* in **g**, **h**, **i**) were myofibroblasts (*red color*, labeled by SMA-alpha in **g**), macrophages (*red color*, labeled by CD68 in **h**), and dendritic cells (*red color*, labeled by S100 in **i**). (**a**–**c**, single IHC, counterstained with hematoxylin, original magnification ×200; **d**–**f**, single IHC, counterstained with hematoxylin, original magnification ×400; **g**–**i**, DIHC, without nuclear counterstaining, original magnification ×400)



sections with ready-to-use Proteinase K solution (Dako, Carpinteria, CA, USA) for 10 min (for IDO IHC) or boiling sections in 0.01 M sodium citrate buffer (pH 6.0) through microwave processing for 15 min (for CD3 IHC), respectively. Then, the sections were incubated with the mouse anti-human IDO monoclonal antibody (working dilution 1:400, Oriental Yeast Co., Ltd., Tokyo, Japan) or anti-CD3 polyclonal antibody (1:50; Dako, Carpinteria, CA, USA)

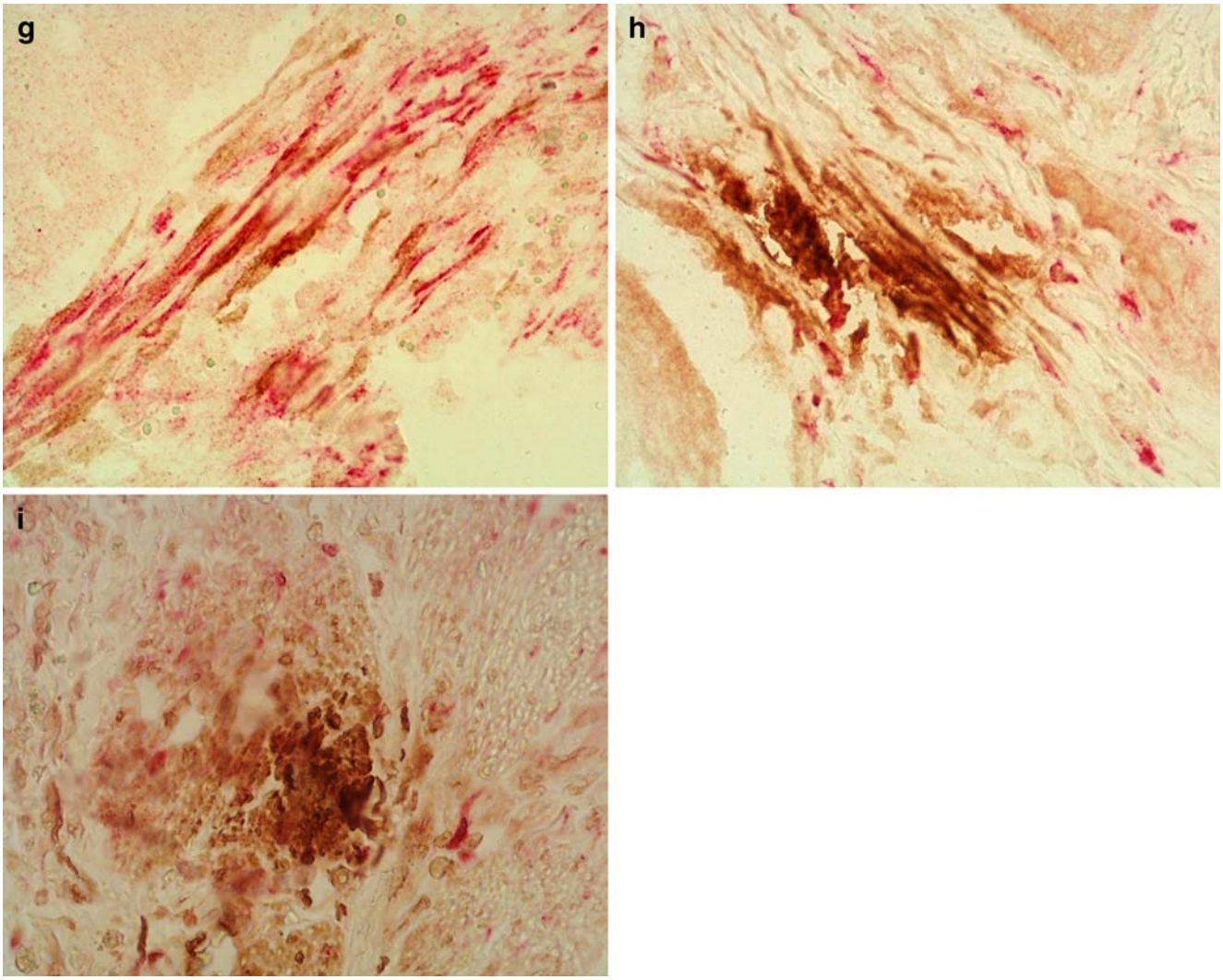


Fig. 1 (continued)

overnight at 4°C. 3-Amino-9-ethylcarbazole (Vector Laboratories, Burlingame, CA, USA) was used as chromogen and Mayer's hematoxylin as the counterstain. The negative control slides were performed routinely: (1) primary antibodies were substituted with the isotype-matched control antibodies; (2) secondary antibody was substituted with phosphate-buffered saline.

Double immunohistochemical identification of cellular phenotypes of IDO in the surrounding noncancerous cells

Since the expression of IDO in cancer cells was easily identified by the specific morphological features, here, we have only examined the cellular phenotypes of IDO in the tumor stromal cells. Double IHCs (DIHCs) were performed using EnVision G72 Doublestain System kit (Dako Demark, Glostrup, Denmark) with antibodies IDO/smooth muscle actin (SMA) alpha (to label myofibroblasts), IDO/CD68 (to label macrophages), and IDO/S100 (to label dendritic cells; monoclonal anti-SMA-alpha, anti-CD68, and anti-S100 antibodies were all purchased from Dako, Carpinteria, CA, USA) according to the manufacturer's instructions and the method described in our publications [25, 26]. In brief, the slides were incubated for 30 min with anti-IDO antibody after antigen retrieval and then incubated with labeled polymer–horseradish peroxidase–antimouse and antirabbit antibodies for 10 min at room temperature. Peroxidase activity was detected with the enzyme substrate 3,3′-diaminobenzidine tetrachloride. After quenching the enzyme reaction, the slides were incubated in Doublestain Block at room temperature for 4 min to block endogenous phosphates. The slides were then incubated with anti-S100, anti-CD68, and anti-SMA-alpha antibodies individually for 15 min at room temperature. After washing, the slides were incubated with rabbit/mouse link

for 10 min and then labeled with polymer–alkaline phosphates antimouse and antirabbit antibodies for 10 min. *Permanent Red* substrate solution was used for the visualization.

Morphometric evaluation

Since the numbers of IDO-positive cells in ESCCs were much lower than in other types of human cancers [10, 13, 15, 20, 27, 28], we used an absolute quantitative method for the IDO-positive density evaluation. The cells with IDO immunoreactivity were counted in malignant epithelium, intratumor stroma, and stroma in invading edges in at least five optical high-power magnification fields (×400) with abundant distribution from each slide. CD3-positive lymphocytes were scored according to the method described in our previous publication [29]. The average values per slide were used for statistical analysis.

Statistical analysis

Results were expressed as mean ± standard error of the mean unless otherwise stated. For statistical analysis of data, the Mann–Whitney tests were used. The level of significance was defined as $P<0.05$.

Results

The expression pattern of IDO in the tumor microenvironment of ESCC

In normal esophageal tissue sections, faint IDO immunoreactivity was occasionally observed in mucosal cells (Fig. 1a; normal controls taken by endoscopic forceps had little stroma; therefore, we could only describe the expression of IDO in mucosal cells). In contrast to the normal controls, the ESCC sections showed intense immunoreactivity for IDO in the cancer epithelium (Fig. 1b) and in the surrounding noncancerous stromal cells as well (Fig. 1c). Most IDO-positive cells were present as clusters and formed a patchy distribution pattern in the cancer epithelium and tumor stroma (Fig. 1b, c). IDO-positive cells in the tumor stroma had various morphological features; many of them were fibroblasts (Fig. 1d), and some were leukocytes (Fig. 1e) and microvessels (Fig. 1f). The intensity of IDO immunoreactivity in tumor cells (Fig. 1b), stromal immune cells (Fig. 1e), and microvessels (Fig. 1f) was stronger than that in fibroblasts (Fig. 1d). Increased IDO expression was confirmed by IDO-positive cell quantification, which showed a significant increase in IDO-positive cell density in the tumor microenvironment as compared with the normal mucosa (see Fig. 2). A particular high density of IDO-positive cells was demonstrated in the invading edges of ESCCs (see Fig. 2). In addition, the IDO-positive cell densities in the compartments of cancer epithelium and tumor stroma were significantly higher than that in the adjacent noncancerous epithelium and nontumor stroma (ESCC vs. adjacent mucosa 0.83±0.23 per field vs. 1.83±0.83 per field, $P\leq0.01$; ESCC tumor stroma vs. adjacent nontumor stroma 3.97±0.73 per field vs. 0.90±0.72 per field, $P\leq0.05$).

The influence of the total IDO-positive cell density on the clinicopathological features in patients with ESCCs was analyzed. The results showed that total IDO-positive cell densities were not significantly correlated with tumor invasion depth, node metastasis, and TNM stages (see Table 2). In addition, statistical analysis showed that the density of CD3-labeled lymphocytes in patients with high IDO cell density (≥10 per field) and with low IDO cell density (≤10 per field) was not different (low IDO cell group vs. high IDO cell group 2.33±0.16 per field vs. 2.59±0.17 per field, $P\geq0.05$).

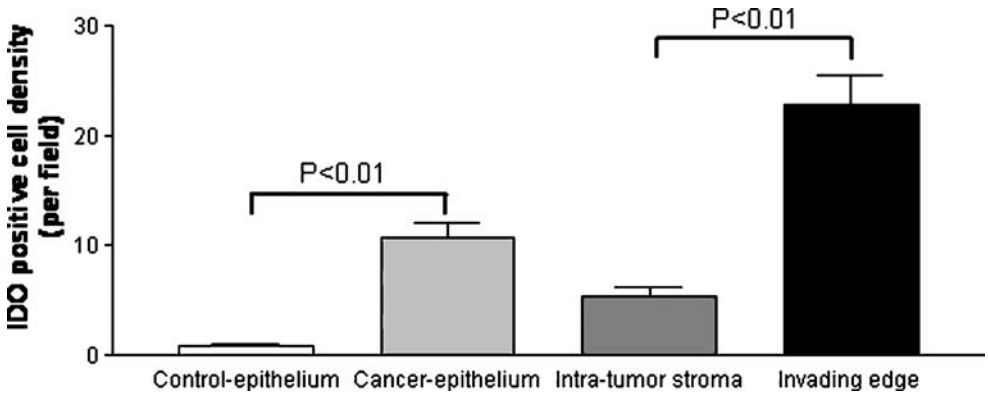


Fig. 2 Graphic analysis of IDO-positive cell densities in ESCCs. The IDO-positive cell density was significantly increased in the ESCC cancer epithelium (*light gray bar*) as compared with the controls (*white bar*); a particular high IDO-positive cell number was observed in the invading edges of ESCCs (*black bar*) as compared with the intratumoral stroma (*dark gray bar*). (The Mann–Whitney test was used for the statistical analysis)

Table 2 The correlation of the total IDO-positive cell density with clinicopathological factors

Factors	Total IDO cell density (field)	*P*
Tumor invasion depth		
T1/T2	12.92±3.66	>0.05
T3	15.25±1.97	
Lymph node involvement		
N0	14.91±1.87	>0.05
N1	18.83±5.37	
TNM stage		
I/II	14.50±1.88	>0.05
III	19.76±4.82	

Cellular phenotypes of IDO in the tumor stroma

The expression of IDO in cancer cells and microvessels can be easily identified by their specific morphological features. However, to identify IDO expression in immune cells (dendritic cells and macrophages) and myofibroblasts in the tumor stroma, DIHCs with specific antibodies (IDO/CD68, IDO/CD, and IDO/SMA alpha) need to be performed. The DIHC results revealed that IDO immunoreactivity could be frequently observed in SMA-alpha-positive myofibroblasts (Fig. 1g), CD68-positive macrophages (Fig. 1h), and S100-positive dendritic cells (Fig. 1i) in the tumor stroma.

Discussion

Immunosuppressive factors derived from the tumor microenvironment play a major role in extending immune escape and facilitating cancer progression. Since the study of Uyttenhove et al. showed that IDO expressed by the tumor cells have an immunoregulatory effect and reduce antitumor T cell attack in a marine tumor model [30], growing evidence from many studies carried out in different human cancers has demonstrated that IDO is expressed by both the tumor cells and nontumor cells in the tumor microenvironment [17]. In the present study, we revealed an increased IDO-positive cell density in the tumor microenvironment of ESCCs and identified that IDO is expressed by tumor cells and many types of cells in the tumor stroma.

IDO is an intracellular enzyme that catalyzes the initial and rate-limiting steps in the metabolism of the essential amino acid tryptophan along the kynurenine pathway [31]. Accumulated evidence has suggested that IDO could be a critical immunosuppressive factor and play an important role in inducing immune tolerance [32, 33]. Our present study has observed an increased number of IDO-positive cells in the microenvironment of ESCCs; these cells were both tumor cells and surrounding noncancerous stromal cells. Quantitative results showed that the IDO-positive cells were ~8 per field in the cancer epithelium and ~5 per field at the site of tumor stroma between cancer epithelium but significantly more common at the site of invading edges (~19 per field). As compared with the reported IDO densities in other types of human cancers [10, 13, 15, 20, 27, 28], IDO-positive cell density in the present study was lower. However, the IDO-positive cell densities were still significantly higher in ESCCs than in controls (see Fig. 2; $P \leq 0.01$). Sakurai et al. have reported that elevated IDO mRNA was found in ESCCs [19]. It is most likely that such increased IDO cell density may represent an increased IDO expression in the tumor microenvironment. In agreement with the findings of Sakurai et al., we also showed that the density of IDO-positive cells did not correlate to tumor invasion, lymph node metastasis, or TNM stage (all $P \geq 0.05$), although the IDO-positive cell densities are slightly increased, paralleling the degree of increase of these parameters. This may be because many factors released from the tumor cells and stroma cells participate in immunosuppression, and IDO only induces immunosuppression to a certain degree [34]. Since the ESCC samples in our study were procured very recently, survival follow-up analysis is not yet available. To further examine IDO-positive cell's prognostic significance, long-term follow-up and large sample studies will be required in the future.

Increased infiltration of immune cells and activated fibroblasts in the tumor stroma is one of the common histological features in ESCCs [35–38]. These cells in the tumor stroma may function as a double-edged sword because they play an important role in both the anticancer response [39, 40] and in favoring cancer progression in some certain conditions [26, 41, 42]. By both the quantitative and qualitative techniques, IDO has been found to be expressed by many types of cells in the tumor microenvironment including tumor cells, fibroblasts, endothelial cells (microvessels), dendritic cells, and macrophages in various cancers [12, 20, 43, 44]. In line with those studies, we were also able to observe IDO immunoreactivity in ESCCs in many types of stromal cells including microvessels, immune cells, and fibroblasts by single IHCs and DIHCs with specific antibodies (see Fig. 1). Those findings suggest that the IDO in ESCCs is most likely from a mixed cellular source, and tumor stroma is an active participant in local immunoregulation.

In addition, the suppressive effect of IDO on T cell function has been reported; we therefore examined the influence of IDO expression on infiltrating lymphocyte number in the tumor microenvironment; however, no statistically significant differences were noted between low and high IDO cell groups ($P > 0.05$). Possible explanations may be: first, T cell's function and number are

regulated by many factors [34]; secondly, T cell contains various subtype groups with different functions [39]; however, the influence of IDO on the different subtype T cells is still unclear; and finally the T cell function changes may be greater than the density changes. Thus, the functional analysis on certain subtype T cell is necessary for the comprehensive understanding of the IDO's effect on host immune functions in ESCCs.

In conclusion, our current findings suggest that increased expression of IDO in ESCCs is from a mixture cellular source in ESCCs and may potentially play an essential role in inducing immune tolerance. Since the use of IDO inhibitors as a possible cancer therapy has been intensively discussed in other types of cancers [45], its potential as a therapeutic application in ESCCs should be investigated in the future.

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

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