TDO2 Overexpression Is Associated with Cancer Stem Cells and Poor Prognosis in Esophageal Squamous Cell Carcinoma

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Keywords
TDO2 · Esophageal squamous cell carcinoma · Spheroid formation · Cancer stem cell · Epidermal growth factor receptor

Abstract
Objective: Esophageal cancer is one of the deadliest cancers in the world, and the main subtype is esophageal squamous cell carcinoma (ESCC), which comprises 90% of cases. Expression of tryptophan 2,3-dioxygenase (TDO2), an enzyme involved in tryptophan catabolism, has been linked with tumor survival and poor prognosis of brain and breast cancer. However, no studies have investigated the potential role of TDO2 in esophageal cancer. Here we explored the expression and biological significance of TDO2 in ESCC. Methods: TDO2 protein expression was evaluated in 90 ESCC tissue samples by immunohistochemistry. TDO2 function in ESCC cell lines and spheroid colony formation were evaluated by RNA interference (RNAi). Results: TDO2 overexpression was associated with tumor stage, recurrence status, and the CD44 cancer stem cell marker in ESCC. TDO2 overexpression was correlated with poor outcome of ESCC patients. Inhibition of TDO2 expression by RNAi in TE-10 and TE-11 cell lines reduced both the number and the size of spheroid colonies as well as cell proliferation. Knockdown of TDO2 expression also induced inactivation of the epidermal growth factor receptor signaling pathway. Conclusion: Our results imply that TDO2 could play an important role in the progression of ESCC. Furthermore, TDO2 may be a potential therapeutic target in ESCC.

Introduction
Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer death worldwide [1]. Esophageal cancer is classified into two main subtypes: esophageal squamous cell carcinoma (ESCC), which ac-
counts for approximately 90% of esophageal cancer cases worldwide, and esophageal adenocarcinoma, which shows increasing rates of incidence and mortality in several regions in North America and Europe [2]. Studies have illustrated the distinction in the molecular characteristics of esophageal adenocarcinoma and ESCC [3]. Patients with esophageal cancer have a poor prognosis, and the 5-year overall survival rate of ESCC patients ranges from 10 to 30% [4, 5]. Targeted therapies for esophageal cancer treatment currently remain limited [6]. Although several clinical trials for targeted treatments of esophageal cancer have been launched, only one study has enrolled patients with ESCC [7]. Therefore, it is urgent to identify new biomarkers and to develop a novel therapeutic target for esophageal cancer.

Cancer stem cells (CSCs) have been reported in many solid tumors [8]. Spheroid colony formation assays, an in vivo technique of plating a limited number of cells in culture dishes specifically coated for nonattachment in serum-free media, have been used to investigate CSC characteristics [9]. We previously analyzed the gene expression profile of spheroid colonies and parental cells derived from gastric cancer (GC) cell lines by microarray analysis [10]. We identified several genes upregulated in spheroid colonies, such as KIF11, KIFC1, and IQGAP3, that are required for spheroid colony formation in GC cell lines and are associated with poor survival of GC patients [10–12]. These genes are also required for spheroid colony formation in esophageal and colorectal cancer cells [13, 14]. Among the genes upregulated in spheroid colonies, tryptophan 2,3-dioxygenase (TDO2) is dramatically upregulated in MKN-1 cells (derived from adenocarcinoma cell carcinoma) compared with other cell lines.

TDO2, indoleamine 2,3-dioxygenase 1 (IDO1), and IDO2 are three enzymes that catalyze the amino acid tryptophan into kynurenine in the kynurenine pathway, which accounts for 95% of tryptophan catabolism [15, 16]. TDO2 is mainly expressed in the liver and exists in the brain, while IDO1 and IDO2 have wider tissue expression, including in peripheral blood and immune cells [16]. TDO2 plays an important role in neurological diseases such as Alzheimer’s disease, Parkinson’s disease, and autism [15, 16]. TDO2 is expressed in several established human cancer cell lines, including glioblastoma, colorectal carcinoma, head and neck carcinoma, and gallbladder carcinoma cells [17]. Overexpression of TDO2 promoted tumor cell survival and was correlated with tumor grade and poor prognosis in triple negative breast cancer [18] and in brain tumors [19]. Furthermore, over-expression of TDO2 has been reported in human colorectal cancer and leiomyosarcoma [20, 21]. However, the expression and biological significance of TDO2 in esophageal cancer have not been investigated.

In this study, we analyzed the expression and distribution of TDO2 in ESCC by immunohistochemistry and examined the relationship between TDO2 expression and clinicopathological characteristics of ESCC. We also evaluated the effect of inhibiting TDO2 expression by RNA interference (RNAi) on spheroid colony formation and cell proliferation.

**Materials and Methods**

**Tissue Samples**

In a retrospective study design, 100 primary tumors were collected from patients diagnosed with ESCC who underwent surgery between 2000 and 2014 at Hiroshima University Hospital, Hiroshima, Japan. All patients underwent curative resection. All patients underwent right transthoracic esophagectomy with extensive lymph node dissection. Reconstruction was performed with a gastric tube positioned in the posterior mediastinum. Adjuvant chemotherapy (low-dose cisplatin and 5-fluorouracil) was performed on all patients [22]. Only patients without preoperative radiotherapy or chemotherapy were enrolled in the study. Operative mortality was defined as death within 30 days of patients leaving the hospital. Postoperative follow-up was scheduled every 1, 2, or 3 months during the first 2 years after surgery and every 6 months thereafter unless more frequent follow-up was deemed necessary. Chest X-ray, chest abdominal computed tomographic scan, and serum chemistry analysis were performed at every follow-up visit. Patients were followed by their physician until death or the date of the last documented contact. Tumor staging was determined according to the TNM classification system [23].

For quantitative reverse transcription polymerase chain reaction (qRT-PCR), we used 10 ESCC samples. Tumor tissues and the corresponding nonneoplastic tissues were surgically removed, frozen immediately in liquid nitrogen, and stored at –80°C until use. A total of 12 types of normal tissue samples were purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA), including heart (catalog No. 636532), lung (catalog No. 636524), stomach (catalog No. 636578), small intestine (catalog No. 636539), colon (catalog No. 636553), liver (catalog No. 636531), pancreas (catalog No. 636577), kidney (catalog No. 636529), bone marrow (catalog No. 636519), leukocytes (catalog No. 636592), skeletal muscle (catalog No. 636547), and brain (catalog No. 636530).

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from the 90 patients who had undergone surgical excision for ESCC. One or two representative tumor blocks, including the tumor center, invading front, and tumor-associated nonneoplastic squamous epithelium, were examined in each patient using immunohistochemistry. In cases of large, late-stage tumors, two different sections were examined to include representative areas of the tumor center as well as in the lateral and deep tumor invasive fronts.
Analysis of TCGA Datasets

To explore the expression of TDO2 in cancer and normal samples, we used an online analytical tool, the Broad Institute TCGA Genome Data Analysis Center [24]. TDO2 was set up as the target gene, and the cohort dataset included liver hepatocellular carcinoma, pancreatic adenocarcinoma, head and neck squamous cell carcinoma, bladder urothelial carcinoma, esophageal carcinoma, colon adenocarcinoma, stomach adenocarcinoma, rectum adenocarcinoma, breast invasive carcinoma, kidney renal clear cell carcinoma, and prostate adenocarcinoma.

qRT-PCR Analysis

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and 1 µg of total RNA was converted to cDNA using the First Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA). Quantitation of TDO2 mRNA level was performed by real-time fluorescence detection as previously described [25]. PCR was conducted using the SYBR Green PCR Core Reagents Kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Real-time detection of the emission intensity of SYBR green bound to double-stranded DNA was performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Actin beta-specific PCR products were amplified from the same RNA samples and served as an internal control. Primer sequences for TDO2 were: forward, 5′-CTG TAT CTA CCA GTG CTC TG-3′; reverse, 5′-CCT TCT GGC ACC ATG-3′. Primer sequences for CD44 were: forward, 5′-TCG TTC AGC ATC TCT CGG ACG GA-3′; reverse, 5′-CAC CCC TTG GTT TGC TG-3′. Primer sequences for actin beta were: forward, 5′-CTG TCT GGC ACC AT-3′; reverse, 5′-GCA ACT AAG TCA TAG TCC GC-3′.

Immunohistochemistry

Immunohistochemical analysis was performed with the EnVision+ Mouse Peroxidase Detection System (Dako Cytomation, Carpinteria, CA, USA) as previously described [26]. A mouse polyclonal anti-TDO2 antibody was used as primary antibody (1:250, catalogue No. H00006999-B01P; Abnova, Taipei, Taiwan). The percentage and the intensity of staining of tumor cells was scored from 0 to 100% and from 0 (no immunoreactivity) to 3+ (intense staining), respectively. The expression score was calculated by the formula A × 1 + B × 2 + C × 3, A representing the percentage of weakly stained cells (score of 1), B representing the percentage of moderately stained cells (score of 2), and C representing the percentage of strongly stained cells (score of 3). The expression score ranged from 0 to 300. Two surgical pathologists, with knowledge of the clinical and pathological parameters of the patients' outcomes, independently reviewed immunoreactivity in each specimen. Interobserver differences were resolved by consensus review at a double-headed microscope after independent review. Immunostaining of CD44 was performed as previously described [26].

Cell Lines

Four ESCC cell lines (TE-1, TE-5, TE-10, and TE-11) were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). All cell lines were maintained in RPMI-1640 (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO2 and 95% air at 37°C.
TDO2 expression level

| TDO2 expression level | Heart | Brain | Lung | Esophagus | Stomach | Small intestine | Liver | Pancreas | Kidney | Bone marrow | Skeletal muscle | Lymphocyte |
|-----------------------|-------|-------|------|-----------|---------|-----------------|-------|----------|--------|-------------|----------------|------------|
| >0.005                |       |       |      |           |         |                 |       |          |        |             |                |            |
| 0.004                 |       |       |      |           |         |                 |       |          |        |             |                |            |
| 0.003                 |       |       |      |           |         |                 |       |          |        |             |                |            |
| 0.002                 |       |       |      |           |         |                 |       |          |        |             |                |            |
| 0.001                 |       |       |      |           |         |                 |       |          |        |             |                |            |
| >0.005                |       |       |      |           |         |                 |       |          |        |             |                |            |
| 0.004                 |       |       |      |           |         |                 |       |          |        |             |                |            |
| 0.003                 |       |       |      |           |         |                 |       |          |        |             |                |            |
| 0.002                 |       |       |      |           |         |                 |       |          |        |             |                |            |
| 0.001                 |       |       |      |           |         |                 |       |          |        |             |                |            |

Fold difference

| Months after surgery | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|----------------------|---|---|---|---|---|---|---|---|---|----|
| Survival probability, % |   |   |   |   |   |   |   |   |   |    |

TDO2 low
(n = 37)

TDO2 high
(n = 53)

log rank p = 0.015

(For legend see next page.)
ences between survival curves were tested for statistical significance by log-rank test. Differences between the two groups (TDO2 siRNA-transfected cells and negative control siRNA-transfected cells) were tested by Student t test.

### Table 1. Relationship between TDO2 expression and clinicopathological characteristics in ESCC

| Characteristic       | TDO2 expression | p value<sup>a</sup> |
|----------------------|-----------------|---------------------|
|                      | high            | low                |
| Age, years           |                 |                     |
| ≤65                  | 26 (62)         | 16 (38)            | 0.586 |
| >65                  | 27 (56)         | 21 (44)            |       |
| Sex                  |                 |                     |
| Female               | 6 (43)          | 8 (57)             | 0.185 |
| Male                 | 47 (62)         | 29 (38)            |       |
| T classification     |                 |                     |
| T1                   | 15 (38)         | 24 (62)            | 0.001 |
| T2/T3/T4             | 38 (75)         | 13 (25)            |       |
| N classification     |                 |                     |
| N0                   | 20 (50)         | 20 (50)            | 0.125 |
| N1/N2/N3             | 33 (66)         | 17 (34)            |       |
| M classification     |                 |                     |
| M0                   | 53 (59)         | 37 (41)            | –      |
| M1                   | 0 (0)           | 0 (0)              |       |
| Stage                |                 |                     |
| I/II                 | 27 (47)         | 31 (53)            | 0.001 |
| III/IV               | 26 (81)         | 6 (19)             |       |
| Histological grade   |                 |                     |
| well/mod.            | 39 (57)         | 30 (43)            | 0.408 |
| poorly               | 14 (67)         | 7 (33)             |       |
| Recurrence           |                 |                     |
| negative             | 35 (51)         | 33 (49)            | 0.012 |
| positive             | 18 (82)         | 4 (18)             |       |

Values for TDO2 expression are presented as n (%). ESCC, esophageal squamous cell carcinoma; mod., moderately; TDO2, tryptophan 2,3-dioxygenase.<sup>a</sup>χ² test.

### Fig. 1. Expression of TDO2 in ESCC. a qRT-PCR analysis of TDO2 mRNA in 10 ESCC samples (T1–T10) and 13 normal tissue samples. The bars represent individual samples. b qRT-PCR of TDO2 in 10 ESCC samples. The bars represent individual samples. Fold difference is the ratio of TDO2 mRNA level in ESCC compared with that in corresponding nonneoplastic tissue. Upregulation was defined as a difference in expression of more than twofold (dashed line). c Immunohistochemical analysis of TDO2 in normal liver. Original magnification ×400. d Immunohistochemical analysis of TDO2 in ESCC. Weak cytoplasmatic staining was observed in normal squamous epithelial cells (arrowhead), whereas strong cytoplasmatic TDO2 staining was detected in ESCC cells (arrow). Original magnification ×20. e Immunohistochemical analysis of TDO2 in ESCC. Strong cytoplasmatic TDO2 staining was detected in ESCC cells. Original magnification ×400. f Kaplan–Meier plot of survival in ESCC patients according to tumor TDO2 expression. ESCC, esophageal squamous cell carcinoma; qRT-PCR, quantitative reverse transcription polymerase chain reaction; TDO2, tryptophan 2,3-dioxygenase.

### Results

#### Expression of TDO2 in ESCC

We first examined the expression of TDO2 in various types of cancers using the TCGA dataset. TDO2 expression was upregulated in most cancers except for liver cancer and pancreas cancer (online suppl. Fig. 1; see www.karger.com/doi/10.1159/000490725 for all online suppl. material). The fold change expression of TDO2 between normal and cancer tissue was highest in esophageal cancer (online suppl. Fig. 1).

We next evaluated the expression of TDO2 in 12 types of normal tissue samples and 10 ESCC tissue samples that contained both tumor tissue (T) and corresponding nonneoplastic tissue (N) using qRT-PCR. Among the 12 normal tissue samples, TDO2 mRNA levels were expressed highest in the liver (Fig. 1a). We also observed high TDO2 mRNA levels in ESCC tissue samples compared with normal tissues. We also calculated the T/N ratios for the 10 ESCC cases and defined upregulation as a change of more than twofold. We found that expression of TDO2 was upregulated in 8 out of the 10 ESCC cases (80%) (Fig. 1b).

Next, we performed immunohistochemistry on 90 ESCC tissue samples, using normal liver tissue as a positive control (Fig. 1c). Normal squamous epithelial cells showed weak staining for TDO2 (Fig. 1d). In contrast, TDO2 expression was increased in ESCC tissues, and TDO2 staining was observed in the cytoplasm of tumor cells (Fig. 1e). Most ESCC cases presented heterogeneity of TDO2 staining, both in the intensity and the percentage of TDO2-stained tumor cells. The percentage and the intensity of staining of tumor cells was calculated as described in Materials and Methods, and representative images are shown in online supplementary Figure 2a. There was no difference in the TDO2 expression score between invasive fronts and the central/superficial areas.

We also used receiver operating characteristic curve analysis to define the cutoff point for the TDO2 expression score correlating with clinicopathological characteristics (online suppl. Fig. 2b–e). Youden’s criterion, which maximizes the sum of sensitivity and specificity, was applied for the optimization cutoff point [29]. By using the Youden index, the cutoff score for T classification, N classification, stage, and recurrence status was 55. TDO2 immunostaining was considered high expression if the expression score was > 55 and TDO2 immunostaining was considered as low expression if the expression score was ≤55. We then examined the relationship of TDO2 expression, classified using the cutoff score, with clinicopathological characteristics of the 90 ESCC patients (Table 1).
Table 2. Uni- and multivariate Cox regression analysis of TDO2 expression and survival in ESCC

| Characteristic                | Univariate analysis |           | Multivariate analysis |           |
|------------------------------|---------------------|-----------|-----------------------|-----------|
|                              | HR (95% CI)         | p value   | HR (95% CI)           | p value   |
| Age                          |                     |           |                       |           |
| ≤65 years                    | 1 (reference)       |           |                       |           |
| >65 years                    | 1.26 (0.48–3.32)    | 0.641     |                       |           |
| Sex                          |                     |           |                       |           |
| Female                       | 1 (reference)       |           |                       |           |
| Male                         | 3.45 (0.50–28.30)   | 0.2       |                       |           |
| T grade                      |                     |           |                       |           |
| T1                           | 1 (reference)       |           | 1 (reference)         |           |
| T2/T3/T4                     | 4.56 (1.31–15.93)   | 0.017     | 0.47 (0.07–3.42)      | 0.458     |
| N grade                      |                     |           |                       |           |
| N0                           | 1 (reference)       |           | 1 (reference)         |           |
| N1/N2                        | 17.42 (2.30–131.92) | 0.006     | 17.68 (1.73–180.76)   | 0.015     |
| Stage                        |                     |           |                       |           |
| I/II                         | 1 (reference)       |           | 1 (reference)         |           |
| III/IV                       | 6.01 (2.11–17.16)   | 0.001     | 1.78 (0.39–8.15)      | 0.459     |
| Histologic grade             |                     |           |                       |           |
| Well/moderately              | 1 (reference)       |           |                       |           |
| Poorly                       | 1.10 (0.38–3.13)    | 0.864     |                       |           |
| Postoperative chemotherapy   |                     |           |                       |           |
| Received                     | 1 (reference)       |           |                       |           |
| Did not receive              | 2.13 (0.81–5.62)    | 0.127     |                       |           |
| TDO2 expression              |                     |           |                       |           |
| Low                          | 1 (reference)       |           | 1 (reference)         |           |
| High                         | 4.17 (1.20–14.57)   | 0.025     | 4.30 (0.98–18.76)     | 0.053     |

CI, confidence interval; ESCC, esophageal squamous cell carcinoma; HR, hazard ratio; TDO2, tryptophan 2,3-dioxygenase.

Fig. 2. Immunohistochemical analysis of TDO2 and CD44 in ESCC. a Immunohistochemical analysis of TDO2. Original magnification ×100. b Immunohistochemical analysis of CD44. Original magnification ×100. ESCC, esophageal squamous cell carcinoma; TDO2, tryptophan 2,3-dioxygenase.
TDO2 high expression was associated with advanced T classification, tumor stage, and recurrence status. These results suggested that TDO2 may play an important role in the progression of ESCC.

Kaplan-Meier analysis further demonstrated that TDO2 high expression ESCC cases showed poorer survival than TDO2 low expression ESCC cases ($p = 0.015$; Fig. 1f). We next performed uni- and multivariate Cox proportional hazards analyses to evaluate the potential role of TDO2 expression as an independent predictor in ESCC (Table 2). In univariate analysis, T classification, N classification, stage, histological classification, and TDO2 expression were associated with poor survival. However, in the multivariate model, only N classification was found to be an independent predictor of survival in ESCC patients (Table 2).

**Relationship between TDO2 Expression and the CSC Marker CD44**

A previous report showed that TDO2 facilitates anoikis resistance in triple-negative breast cancer [18], and another study indicated that resistance to anoikis was related to CSC-like cells [30]. Thus, we hypothesized that TDO2 expression may be related to CSC marker expression in ESCC. Numerous studies have identified CSC markers in esophageal cancer, such as CD44, ALDH1, and CD133, and the pathways involved in CSCs, such as Hippo, Wnt/β-catenin, and Notch [31]. Among these CSC markers, CD44 is a well-known CSC marker in esophageal cancer [31]. We thus performed immunohistochemical analysis of CD44 in 90 ESCC cases. In normal squamous epithelium, CD44 displayed uniform membranous staining in basal cell layers. Interestingly, we found that TDO2-expressing cancer cells also expressed CD44 (Fig. 2). Furthermore, ESCCs with high TDO2 expression showed significantly enriched numbers of CD44-positive cells (Table 3).

**Effect of TDO2 Inhibition on Spheroid Formation**

The immunohistochemical results indicated that CD44-positive ESCC cases showed increased expression of TDO2. However, the significance of TDO2 expression in ESCC CSCs was unknown. Therefore, we next investigated the effect of inhibiting TDO2 expression on spheroid colony formation. We first established spheroid body-forming cells from ESCC cell lines. qRT-PCR revealed that spheroid body-forming cells showed enriched CD44 expression in TE-5, TE-10, and TE-11 cell lines (online suppl. Fig. 3a). We then measured the expression of TDO2 mRNA in spheroid body-forming cells from ESCC cell lines and the parental cells. TDO2 mRNA levels were dramatically upregulated in spheroid body-forming cells compared with the parental cells in all four ESCC cell lines (Fig. 3a). We next examined the effect of TDO2 inhibition by siRNA transfection on sphere number and size. We selected TE-10 and TE-11 cells, which exhibited high levels of TDO2 mRNA expression in spheroid body-forming and parental cells, for analysis. Two different siRNAs targeting TDO2 were transfected into TE-10 and TE-11 cells, and TDO2 mRNA expression was successfully suppressed by siRNA1 and siRNA2 transfection (Fig. 3b; online suppl. Fig. 3b). We next evaluated the impact of TDO2 knockdown by evaluating the number and size of spheres at day 15 after transfection. TE-11 and TE-10 cells transfected with TDO2 siRNA showed reduced number and size of spheres compared with negative control transfected cells (Fig. 3c; online suppl. Fig. 3c). These results suggest that TDO2 is required for spheroid colony formation in ESCC.

**Effect of TDO2 Inhibition on Cell Growth and Invasive Activities**

We next examined the effect of TDO2 inhibition on cell growth using MTT assays. As shown in Figure 4a and online supplementary Figure 4a, TDO2 siRNA1- and siRNA2-transfected TE-11 and TE-10 cells showed significantly reduced cell growth compared with negative control siRNA-transfected TE-11 and TE-10 cells ($p < 0.05$).

To determine the possible role of TDO2 in the invasiveness of ESCC cells, we used modified Boyden chamber assays. On day 2, the invasiveness of TDO2 siRNA1- and siRNA2-transfected TE-11 and TE-10 cells was less than that of the negative control siRNA-transfected cells ($p < 0.05$; Fig. 4b and online suppl. Fig. 4b). However, as TDO2 knockdown cells showed significantly reduced cell growth, the differences in cell number observed in the invasion assay could be caused by the reduced cell growth.

### Table 3. Relationship between TDO2 expression and CD44 expression

| CD44       | TDO2 expression | $p$ value$^a$ |
|------------|-----------------|---------------|
| positive   | high            | 49 (64)       |
| negative   | low             | 28 (36)       |
|            | high            | 4 (31)        |
|            | low             | 9 (69)        |

Values for TDO2 expression are presented as $n$ (%). TDO2, tryptophan 2,3-dioxygenase. $^a$χ² test.
These results suggest that TDO2 stimulates cell growth and invasion in cancer cells.

We also examined the effect of TDO2 inhibition on drug resistance. Since 5-fluorouracil is an important treatment option for patients with ESCC, resistance to 5-fluorouracil was examined in the present study. MTT assays were performed to measure the cell viability of TDO2 siRNA- and negative control siRNA-transfected cells under various concentrations of docetaxel for 48 h. However, the 50% inhibitory concentration value of TDO2 siRNA1- and siRNA2-transfected TE-11 and TE-10 cells was not significantly different (data not shown).

EGFR activates the RAS-MEK-ERK and AKT-PI3K pathways, leading to cancer cell proliferation and survival [32]. Additionally, kynurenine is the product of downstream metabolites of tryptophan by TDO2 and acts as
both a regulatory molecule and endogenous ligand for the aryl hydrocarbon receptor (AhR) [19]. A previous report demonstrated that the crosstalk between AhR and EGFR is involved in regulating colon cancer cell proliferation [33]. Therefore, we next analyzed the effect of TDO2 inhibition on the EGFR signaling pathway. Western blot analysis confirmed successful TDO2 knockdown in TE-11 and TE-10 cells transfected with TDO2 siRNA (Fig. 4c; online suppl. Fig. 4c). We also found that the levels of phosphorylated EGFR, Erk, and Akt were lower in TDO2 siRNA1- and siRNA2-transfected TE-11 and TE-10 cells compared with control cells (Fig. 4b; online suppl. Fig. 4b).

Discussion

CSCs play an essential role in tumor progression, metastasis, and cancer recurrence, as well as resistance to chemotherapy and radiation therapy [8]. We previously identified the expression of CD44 and two other CSC markers, ALDH1 and CD133, in GC and found that CD44 is an independent prognosis marker for GC patients [26]. In ESCC, CD44 expression is a predictive marker in patients treated with neoadjuvant chemotherapy after radical esophagectomy [34]. In the current study, we found that the overexpression of TDO2 was associated with advanced disease and poor outcome of ESCC patients. Additionally, the expression of TDO2 was correlated with CD44 expression in ESCC tumor tissues. Inhibition of TDO2 expression decreased the number and size of spheroid colonies in ESCC cell lines. Together our results reveal that TDO2 expression may play an indispensable role in ESCC stem cells.

Immune-targeted therapy is now becoming a new strategy for cancer treatment, with various drugs approved by the Food and Drug Administration [35]. A previous report showed that tryptophan metabolism by IDO1 mediated tumor immune tolerance, and an in...
hibitor targeting IDO1 is currently under investigation in a clinical trial [36]. Another study using an immunized mouse model showed that tumors acquired immune tolerance by inducing TDO2 expression [17]. An inhibitor of TDO2 was previously developed and has been used in in vitro studies [37]. Here we show that TDO2 may play a crucial role in cancer cell survival, as inhibition of TDO2 by siRNA suppressed ESCC cell line proliferation. Research in brain tumors corroborates our data, and TDO2 expression not only suppresses antitumor immune responses, but also promotes tumor cell survival [19]. Furthermore, IDO1 was reported to play a role in the progression of ESCC and to serve as a predictor for poor prognosis in ESCC [38]. Together these data indicate that the relationship between TDO2 expression and IDO1 expression and the tumor immune response in ESCC should be analyzed in future studies.

TDO2 catalyzes tryptophan into kynurenine in the kynurenine pathway [15, 16]. It is well known that tryptophan derivatives such as 6-formylindolo[3, 2-b]carbazole, kynurenine, and 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) are natural ligands of the AhR [39]. The synthetic agonist of the AhR can downregulate the master pluripotency factor Oct4 in stem-like breast cancer cell lines and inhibit their proliferation and metastasis [39], indicating that synthetic ITE can induce the differentiation of stem-like cancer cells and reduces their tumorigenic potential. In fact, it has been reported that reduction of endogenous ITE levels in cancer cells by tryptophan deprivation leads to elevation of Oct4, which is the master pluripotency factor [39]. Therefore, overexpression of TDO2 could reduce the tryptophan level in cancer cells and induce Oct4 expression.

EGFR overexpression is frequently observed in 50% of ESCCs [40]. A clinical phase II trial in advanced ESCC showed that the combination of cetuximab, an EGFR-blocking monoclonal antibody, with cisplatin/5-fluorouracil increased the efficacy of standard chemotherapy [41]. Our results showed that the EGFR signaling pathway was dramatically inactivated by the inhibition of TDO2 expression in ESCC. These findings indicate that TDO2 could participate in the activation of EGFR, and they imply that EGFR signaling may be important for ESCC cell growth in TDO2 high expression ESCCs.

Interestingly, a previous study reported that inhibition of EGFR could prevent the induction of cancer stem-like cells in ESCC through suppressing epithelial-mesenchymal transition [42]. Recent research has revealed the correlation between cancer stemness and epithelial-mesenchymal transition [42, 43]. In this study we showed that transient knockdown of TDO2 by RNAi reduced activation of the EGFR signaling pathway and reduced spheroid formation in ESCC cell lines, although the epithelial-mesenchymal transition profiles were not examined. Therefore, further research is required for the specificity of TDO2.

Our study has several limitations. It is a retrospective study. Since retrospective studies are highly supportive of a result, we need additional retrospective studies. Furthermore, the correlation between expression of TDO2 and EGFR or Oct4 should be analyzed in the near future.

In summary, in this study we found that TDO2 overexpression was related to poor prognosis and associated with cancer cell proliferation and CSCs in ESCC. Suppression of TDO2 inhibited activation of the EGFR signaling pathway and spheroid formation. Our data indicate that TDO2 inhibition may be an essential target for clinical trial research in ESCC.

**Statement of Ethics**

This study was approved (No. IRINHI66) by the Ethics Committee for Human Genome Research of Hiroshima University, Hiroshima, Japan.

**Disclosure Statement**

The authors report no potential conflicts of interest.

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