TDO2 Overexpression Is Associated With Cancer Stem Cells And Poor Prognosis In Esophageal Squamous Cell Carcinoma

食道扁平上皮癌における TDO2 の過剰発現は癌幹細胞および不良な予後と関連する

PHAM QUOC THANG

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 invested 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 was 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 EGFR 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 accounts for approximately 90% of esophageal cancer cases worldwide, and esophageal adenocarcinoma (EAC), 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 EAC and ESCC [3]. Patients with esophageal cancer have 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 launched, only one study has enrolled patients with ESCC [7]. Therefore, it is urgent to identify new biomarkers and 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 cells in culture dishes specifically coated for non-attachment 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 adenosquamous 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, overexpression 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 clinicopathologic 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 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]. This study was approved (No. IRINHI66) by the Ethical Committee for Human Genome Research of Hiroshima University (Hiroshima, Japan).

For quantitative reverse transcription-polymerase chain reaction (qRT-PCR), we used 10 ESCC samples. Tumor tissues and the corresponding non-neoplastic tissue 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. (Mountainview, 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. 636591), 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 non-neoplastic squamous epithelial, 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, http://firebrowse.org/ [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 (ACTB)-specific PCR products were amplified from the same RNA samples and served as an internal control. Primer sequences for TDO2 were forward, 5′-CGG TGG TTC CTC AGG CTA TC-3′ and reverse, 5′-CTT CGG TAT CCA GTG TCG GG-3′. Primer sequences for CD44 were forward, 5′-TAC AGC ATC TCT CGG ACG GA-3′ and reverse, 5′-CAC CCC TGT GTT GTT TGC TG-3′. Primer sequences for ACTB were forward, 5′-CTG TCT GGC GGC ACC ACC AT-3′ and 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 [23]. A mouse polyclonal anti-TDO2 antibody was used as primary antibody (1:250; catalogue no. H00006999-B01P, Abnova, Jhouzih St., Taipei, Taiwan). The percentage and the intensity of staining of tumor cells was scored from 0–100% and from 0 (no immunoreactivity) to 3+ (intense staining), respectively. The expression score was calculated by the formula: Ax1+Bx2+Cx3, in which A represents the percentage of weakly stained (score of 1) cells, B represents the percentage of moderately stained (score of 2) cells, and C represents the percentage of strongly stained (score of 3) cells. The expression score ranged from 0 to 300. Two surgical pathologists, without knowledge of the clinical and pathologic parameters or 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% CO_2 and 95% air at 37°C.

Western blot analysis

Cells were lysed as previously described [27]. The lysates (40 μg) were solubilized in Laemmli sample buffer by boiling and then subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by electrotransfer onto a nitrocellulose filter. Western blot procedures were performed as previously described [27]. Anti-TDO2 antibody was purchased from Abnova. Anti-epidermal growth factor receptor (EGFR), anti-phospho-EGFR (pEGFR), anti-Erk, anti-phospho-Erk1/2 (pErk1/2), anti-Akt, and anti-phospho-Akt (pAkt) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Western Blot Detection System (Amersham Biosciences). β-Actin (Sigma) was used as a loading control.

RNAi

Short interfering RNA (siRNA) oligonucleotides targeting TDO2 and a negative control were purchased from Invitrogen (Carlsbad, CA, USA). We used two independent TDO2 siRNA oligonucleotide sequences (catalog no. 10620318 and 10620319). Transfection was performed using Lipofectamine RNAiMAX (Invitrogen) as previously described [25]. Briefly, 60 pmol of siRNA and 10 μl of Lipofectamine RNAiMAX were mixed in 1 ml of

RMPI medium (10 nmol/l final siRNA concentration). After 20 min of incubation, the mixture was added to cells and then cells were plated in culture dishes. Forty-eight hours after transfection, cells were analyzed.

Cell growth assays

We performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays as previously described [28]. The cells were seeded at a density of 2,000 cells per well in 96-well plates. Cell growth was examined after 1, 2, and 4 days. Three independent experiments were performed. The mean and standard deviation (SD) were calculated for each experiment.

Spheroid colony formation assay

For the generation of spheres, 2,000 cells were plated in each well of 24-well ultra-low attachment plates (Corning) containing mTeSR medium (STEMCELL Technologies Inc., Cambridge, MA, USA). The plates were incubated at 37°C in a 5% CO_2 incubator for 15 days. Sphere number and size were determined and counted under a microscope.

Statistical methods

Associations between clinicopathological parameters and TDO2 expression were analyzed by Chi-Squared test. Kaplan–Meier survival curves were constructed for TDO2 high expression and TDO2 low expression patients. Survival rates were compared between TDO2 high expression and TDO2 low expression groups. Differences between survival curves were tested for statistical significance by a log-rank test. Differences between the two groups (TDO2 siRNA-transfected cells and negative control siRNA-transfected cells) were tested by Student t-test.

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 (**Supplementary Fig. 1**). The fold change expression of TDO2 between normal and cancer tissue was the highest in esophageal cancer (**Supplementary 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 non-neoplastic tissue (N) using qRT-PCR. Among the 12 normal tissue samples, TDO2 mRNA levels were expressed the highest in 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 two-fold. 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 the Methods section and representative images are shown in **Supplementary Fig. 2a**. There was no difference in the TDO2 expression score between invasive fronts and the central/superficial areas.

We also used receiver operating characteristic (ROC) curve analysis to define the cut-off point for the TDO2 expression score correlating with clinicopathological

characteristics (**Supplementary Fig. 2b–e**). Youden's criterion, which maximizes the sum of sensitivity and specificity, was applied for the optimization cut-off point [29]. By using the Youden's index, the cut-off score for T classification, N classification, stage and recurrence status was 55. TDO2 immunostaining was considered high expression if the expression score was over 55 and TDO2 immunostaining was considered as low expression if the expression score was below or equal to 55. We then examined the relationship of TDO2 expression, classified using the cut-off score, with clinicopathological characteristics of the 90 ESCC patients (**Table 1**). 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 univariate 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**).

Relation 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 with CSC-like cells [30]. Thus, we hypothesized that TDO2 expression may be related with 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 **(Supplementary 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, Supplementary 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, Supplementary Fig. 3c**). These results suggest that TDO2 is required for spheroid colony formation in ESCC.

TDO2 inhibition reduced cell growth and mediated EGFR pathway signaling

We next examined the effect of TDO2 inhibition on cell growth using MTT assays. As shown in **Fig. 4a** and **Supplementary Fig. 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 ($p < 0.05$). We also examined the effect of TDO2 inhibition on drug resistance. Since 5-fluorouracil is an important treat- ment 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 5-FU 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 (**Supplementary Fig. 5a-b**).

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. 4b, Supplementary Fig. 4b**). 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, Supplementary 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 inhibitor 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 anti-tumor immune responses and but also promotes tumor cell survival [19]. Furthermore, IDO1 was reported to play a role in the progression of ESCC and 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 tryp- tophan derivatives such as 6-formylindolo[3, 2-b]car- bazole, 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 trial phase II in advanced ESCCs 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 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 (EMT) [42]. Recent research has revealed the correlation between cancer stemness and EMT [42,439]. 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 EMT profiles were not examined. Therefore, further research is required for the specificity of TDO2.

In summary, in this study, we found that TDO2 overexpression was related with a

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 indicates 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 Ethical Committee for Human Genome Research of Hiroshima University (Hiroshima, Japan).

Disclosure Statement

The authors report no potential conflicts of interest.

Funding Sources

The present study was supported by Grants-in-Aid for Scientific Research (B-15H04713) and Challenging Exploratory Research (grant nos. 26670175 and 16K15247) (both from the Japan Society for the Promotion of Science) and by the Takeda Science Foundation.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F: Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136(5):E359-86.

2. Rustgi AK, El-Serag HB: Esophageal carcinoma. N Engl J Med 2014;371(26):2499-509.

3. Montgomery E, Field JK, Boffetta P, Daigo Y, Shimizu M, Shimoda T: Squamous cell carcinoma of the oesophagus; In: Bosman FT, Carneiro F, Hruban RH, Theise ND (Eds): WHO Classification of Tumors of the Digestive System, IARC, Lyon, 2010, pp 18-24.

4. Morita M, Yoshida R, Ikeda K, Egashira A, Oki E, Sadanaga N, Kakeji Y, Yamanaka T, Maehara Y: Advances in esophageal cancer surgery in Japan: an analysis of 1000 consecutive patients treated at a single institute. Surgery 2008;143(4):499-508.

5. The Cancer Genome Atlas Research Network: Integrated genomic characterization of oesophageal carcinoma. Nature 2017;541(7636):169-175.

6. National Comprehensive Cancer Network: Clinical Practice Guidelines in Oncology. Esophageal and esophagogastric junction cancers, version 1.2017. https://www.nccn.org/professionals/physician_gls/pdf/esophageal.pdf (accessed July 30th, 2017).

7. Smyth EC, Lagergren J, Fitzgerald RC, Lordick F, Shah MA, Lagergren P, Cunningham D: Oesophageal cancer. Nat Rev Dis Primers 2017;3:17048.

8. Batlle E, Clevers H: Cancer stem cells revisited. Nat Med 2017;23(10):1124-1134.

9. Takaishi S, Okumura T and Wang TC: Gastric cancer stem cells. J Clin Oncol 2008;26(17):2876-82.

10. Oue N, Mukai S, Imai T, Pham TT, Oshima T, Sentani K, Sakamoto N, Yoshida K, Yasui W: Induction of KIFC1 expression in gastric cancer spheroids. Oncol Rep

2016;36(1):349-55.

11. Oue N, Yamamoto Y, Oshima T, Asai R, Ishikawa A, Uraoka N, Sakamoto N, Sentani K, Yasui W: Overexpression of the Transmembrane Protein IQGAP3 Is Associated with Poor Survival of Patients with Gastric Cancer. Pathobiology, Epub ahead of print.

12. Imai T, Oue N, Nishioka M, Mukai S, Oshima T, Sakamoto N, Sentani K, Matsusaki K, Yoshida K, Yasui W: Overexpression of KIF11 in Gastric Cancer with Intestinal Mucin Phenotype. Pathobiology 2017;84(1):16-24.

13. Imai T, Oue N, Yamamoto Y, Asai R, Uraoka N, Sentani K, Yoshida K, Yasui W: Overexpression of KIFC1 and its association with spheroid formation in esophageal squamous cell carcinoma. Pathol Res Pract 2017;213(11):1388-1393.

14. Imai T, Oue N, Sentani K, Sakamoto N, Uraoka N, Egi H, Hinoi T, Ohdan H, Yoshida K, Yasui W: KIF11 Is Required for Spheroid Formation by Oesophageal and Colorectal Cancer Cells. Anticancer Res 2017;37(1):47-55.

15. Platten M, Wick W, Van den Eynde BJ: Tryptophan catabolism in cancer: beyond IDO and tryptophan depletion. Cancer Res 2012;72(21):5435-40.

16. Badawy AA: Kynurenine Pathway of Tryptophan Metabolism: Regulatory and Functional Aspects. Int J Tryptophan Res DOI: 10.1177/1178646917691938.

17. Pilotte L, Larrieu P, Stroobant V, Colau D, Dolusic E, Frédérick R, De Plaen E, Uyttenhove C, Wouters J, Masereel B, Van den Eynde BJ: Reversal of tumoral immune resistance by inhibition of tryptophan 2,3-dioxygenase. Proc Natl Acad Sci U S A 2012;109(7):2497-502.

18. D'Amato NC, Rogers TJ, Gordon MA, Greene LI, Cochrane DR, Spoelstra NS, Nemkov TG, D'Alessandro A, Hansen KC, Richer JK: A TDO2-AhR signaling axis facilitates anoikis resistance and metastasis in triple-negative breast cancer. Cancer Res 2015;75(21):4651-64.

19. Opitz CA, Litzenburger UM, Sahm F, Ott M, Tritschler I, Trump S, Schumacher T, Jestaedt L, Schrenk D, Weller M, Jugold M, Guillemin GJ, Miller CL, Lutz C, Radlwimmer B, Lehmann I, von Deimling A, Wick W, Platten M: An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. Nature 2011;478(7368):197-203.

20. Puccetti P, Fallarino F, Italiano A, Soubeyran I, MacGrogan G, Debled M, Velasco V, Bo- det D, Eimer S, Veldhoen M, Prendergast GC, Platten M, Bessede A, Guillemin GJ: Accumulation of an endogenous tryptophan derived metabolite in colorectal and breast cancers. PLoS One 2015;10:e0122046.

21. Cuppens T, Moisse M, Depreeuw J, Anniba- li D, Colas E, Gil-Moreno A, Huvila J, Car- pén O, Zikán M, Matias-Guiu X, Moerman P, Croce S, Lambrechts D, Amant F: Integrated genome analysis of uterine leiomyosarcoma to identify novel driver genes and targetable pathways. Int J Cancer 2018;142: 1230–1243.

22. Shimizu K, Hihara J, Yoshida K, Toge T: Clinical evaluation of low-dose cisplatin and 5-fluorouracil as adjuvant chemoradiothera- py for advanced squamous cell carcinoma of the esophagus. Hiroshima J Med Sci 2005;54: 67–71.

23. Sobin LH, Gospodarowicz MK, Wittekind CH: TMN classification of malignant tumors, ed 7th, Wiley-Liss, New York, 2009, pp 66-72.

24. Broad Institute TCGA Genome Data Analysis Center, http://firebrowse.org/viewGene.html?gene=tdo2 (accessed July 30th, 2017).

25. Sakamoto N, Oue N, Sentani K, Anami K, Uraoka N, Naito Y, Oo HZ, Hinoi T, Ohdan H, Yanagihara K, Aoyagi K, Sasaki H, Yasui W: Liver-intestine cadherin induction by epidermal growth factor receptor is associated with intestinal differentiation of gastric cancer. Cancer Sci 2012;103(9):1744-50.

26. Wakamatsu Y, Sakamoto N, Oo HZ, Naito Y, Uraoka N, Anami K, Sentani K, Oue N, Yasui W: Expression of cancer stem cell markers ALDH1, CD44 and CD133 in primary

tumor and lymph node metastasis of gastric cancer. Pathol Int 2012;62(2):112-9.

27. Yasui W, Ayhan A, Kitadai Y, Nishimura K, Yokozaki H, Ito H, Tahara E: Increased expression of p34cdc2 and its kinase activity in human gastric and colonic carcinomas. Int J Cancer 1993:53(1):36-41.

28. Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, Boyd MR: Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res 1988;48(3):589-601.

29. Perkins NJ, Schisterman EF: The Inconsistency of "Optimal" Cut-points Using Two ROC Based Criteria. Am J Epidemiol 2006;163(7):670-5.

30. Kim SY, Hong SH, Basse PH, Wu C, Bartlett DL, Kwon YT, Lee YJ: Cancer Stem Cells Protect Non-Stem Cells From Anoikis: Bystander Effects. J Cell Biochem 2016;117(10):2289-301.

31. Wang D, Plukker JTM, Coppes RP: Cancer stem cells with increased metastatic potential as a therapeutic target for esophageal cancer. Semin Cancer Biol 2017;44:60-66.

32. Li S, Schmitz KR, Jeffrey PD, Wiltzius JJ, Kussie P, Ferguson KM: Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. Cancer Cell 2005;7(4):301-11.

33. Xie G, Peng Z, Raufman JP: Src-mediated aryl hydrocarbon and epidermal growth factor receptor cross talk stimulates colon cancer cell proliferation. Am J Physiol Gastrointest Liver Physiol 2012;302(9):G1006-15.

34. Okamoto K, Ninomiya I, Ohbatake Y, Hirose A, Tsukada T, Nakanuma S, Sakai S, Kinoshita J, Makino I, Nakamura K, Hayashi H, Oyama K, Inokuchi M, Nakagawara H, Miyashita T, Hidehiro T, Takamura H, Fushida S, Ohta T: Expression status of CD44 and CD133 as a prognostic marker in esophageal squamous cell carcinoma treated with neoadjuvant chemotherapy followed by radical esophagectomy. Oncol Rep 2016;36(6):3333-

3342.

35. Shekarian T, Valsesia-Wittmann S, Caux C, Marabelle A: Paradigm shift in oncology: targeting the immune system rather than cancer cells. Mutagenesis 2015;30(2):205-11.

36. Munn DH, Mellor AL: IDO in the Tumor Microenvironment: Inflammation, Counter-Regulation, and Tolerance. Trends Immunol 2016;37(3):193-207.

37. Pantouris G, Mowat CG: Antitumour agents as inhibitors of tryptophan 2,3-dioxygenase. Biochem Biophys Res Commun 2014;443(1):28-31.

38. Jia Y, Wang H, Wang Y, Wang T, Wang M, Ma M, Duan Y, Meng X, Liu L: Low expression of Bin1, along with high expression of IDO in tumor tissue and draining lymph nodes, are predictors of poor prognosis for esophageal squamous cell cancer patients. Int J Cancer 2015;137(5):1095-106.

39. Cheng J, Li W, Kang B, Zhou Y, Song J, Dan S, Yang Y, Zhang X, Li J, Yin S, Cao H, Yao H, Zhu C, Yi W, Zhao Q, Xu X, Zheng M, Zheng S, Li L, Shen B, Wang YJ: Tryptophan derivatives regulate the transcription of Oct4 in stem-like cancer cells. Nat Commun 2015; 6:7209

40. Hanawa M, Suzuki S, Dobashi Y, Yamane T, Kono K, Enomoto N, Ooi A: EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus. Int J Cancer 2006;118(5):1173-80.

41. Lorenzen S, Schuster T, Porschen R, Al-Batran SE, Hofheinz R, Thuss-Patience P, Moehler M, Grabowski P, Arnold D, Greten T, Müller L, Röthling N, Peschel C, Langer R, Lordick F: Cetuximab plus cisplatin-5-fluorouracil versus cisplatin-5-fluorouracil alone in first-line metastatic squamous cell carcinoma of the esophagus: a randomized phase II study of the Arbeitsgemeinschaft Internistische Onkologie. Ann Oncol 2009;20(10):1667-73.

Figure legends

Fig. 1. Expression of TDO2 in ESCC. (a) Quantitative reverse transcription-polymerase chain reaction (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 non-neoplastic tissue. Upregulation was defined as a difference in expression of more than two-fold (dotted line). (c) Immunohistochemical analysis of TDO2 in normal liver. Original magnification, x400. (d) Immunohistochemical analysis of TDO2 in ESCC. Weak cytoplasmic staining was observed in normal squamous epithelial cells (arrowhead), whereas strong cytoplasmic TDO2 staining was detected in ESCC cells (arrow). Original magnification, x20. (e) Immunohistochemical analysis of TDO2 in ESCC. Strong cytoplasmic TDO2 staining was detected in ESCC cells. Original magnification, x400. (f) Kaplan–Meier plot of survival in ESCC patients according to tumor TDO2 expression.

Fig. 2. Immunohistochemical analysis of TDO2 and CD44 in ESCC. (a) Immunohistochemical analysis of TDO2. Original magnification, x100. (b) Immunohistochemical analysis of CD44. Original magnification, x100.

Fig. 3. Effect of TDO2 inhibition on spheroid formation. (a) qRT-PCR analysis of TDO2 mRNA levels in spheroid body-forming cells and parental cells in ESCC cell lines. (b) qRT-PCR analysis of TDO2 mRNA in TE-11 cells transfected with two siRNAs targeting TDO2. (c) The number and size of spheres from TE-11 cells transfected with TDO2 siRNA or negative control siRNA. Bars and error bars indicate mean ± SD, respectively, of three different experiments. Scale bar, 100 μm. *$p < 0.05$.

Fig. 4. Effect of TDO2 inhibition on cell growth in TE-11 cells. (a) Effect of TDO2 knockdown on cell growth of TE-11 cells. Cell growth was assessed by MTT assays at 1, 2, and 4 days after seeding TDO2 siRNA-transfected TE-11 cells on 96-well plates. Bars and error bars indicate the mean and standard error (SE). (b) Western blot analysis of TDO2, EGFR, phospho-EGFR (pEGFR), Erk1/2, phospho-Erk1/2 (pErk1/2), Akt, and phospho-Akt (pAkt) in cell lysates from TDO2 cells transfected with TDO2 siRNAs or negative control siRNA. β-Actin was included as a loading control. *$p < 0.05$.

Table 1. Relationship between TDO2 expression and clinicopathological characteristics in esophageal squamous cell carcinoma.

		TDO2 expression, n (%)		
		High	Low	*p* value[a]
Age	≤ 65	26(62)	16	0.586
	> 65	27(56)	21	
Sex	Female	6(43)	8	0.185
	Male	47(62)	29	
T classification	T1	15(38)	24	0.001
	T2/3/4	38(75)	13	
N classification	N0	20(50)	20	0.125
	N1/2/3	33(66)	17	
M classification	M0	53(59)	37	-
	M1	0(-)	0(-)	
Stage	Stage I/II	27(47)	31	0.001
	Stage III/IV	26(81)	6	
Histological grade	Well/moderately	39(57)	30	0.408
	Poorly	14(67)	7	
Recurrence	Negative	35(51)	33	0.012
	Positive	18(82)	4	

[a] Chi-squared test

Table 2. Univariate and multivariate Cox regression analysis of TDO2 expression and survival in esophageal squamous cell carcinoma.

	Univariate analysis		Multivariate analysis	
Characteristic	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Age				
≤ 65	1 (Ref.)			
> 65	1.26 (0.48-3.32)	0.641		
Sex				
Female	1 (Ref.)			
Male	3.45 (0.50-28.30)	0.2		
T grade				
T1	1 (Ref.)		1 (Ref.)	
T2/T3/T4	4.56 (1.31-15.93)	0.017	0.47 (0.07-3.42)	0.458
N grade				
N0	1 (Ref.)		1 (Ref.)	
N1/2	17.42 (2.30-131.92)	0.006	17.68 (1.73-180.76)	0.015
Stage				
Stage I/II	1 (Ref.)		1 (Ref.)	
Stage III/IV	6.01 (2.11-17.16)	0.001	1.78 (0.39-8.15)	0.459
Histologic grade				
Well/moderately	1 (Ref.)			
Poorly	1.10 (0.38-3.13)	0.864		
Postoperative chemotherapy				
Received	1 (Ref.)			
Did not received	2.13 (0.81-5.62)	0.127		

TDO2 expression				
Low	1 (Ref.)		1 (Ref.)	
High	4.17 (1.20-14.57)	0.025	4.30 (0.98-18.76)	0.053

HR: hazard ratio, CI: confidence interval.

Table 3. Relationship between TDO2 expression and CD44 expression.

		TDO2 expression, n (%)		
		High	Low	*p* value[a]
CD44	Positive	49(64)	28	0.026
	Negative	4(31)	9	

[a] Chi-squared test

Fig.1

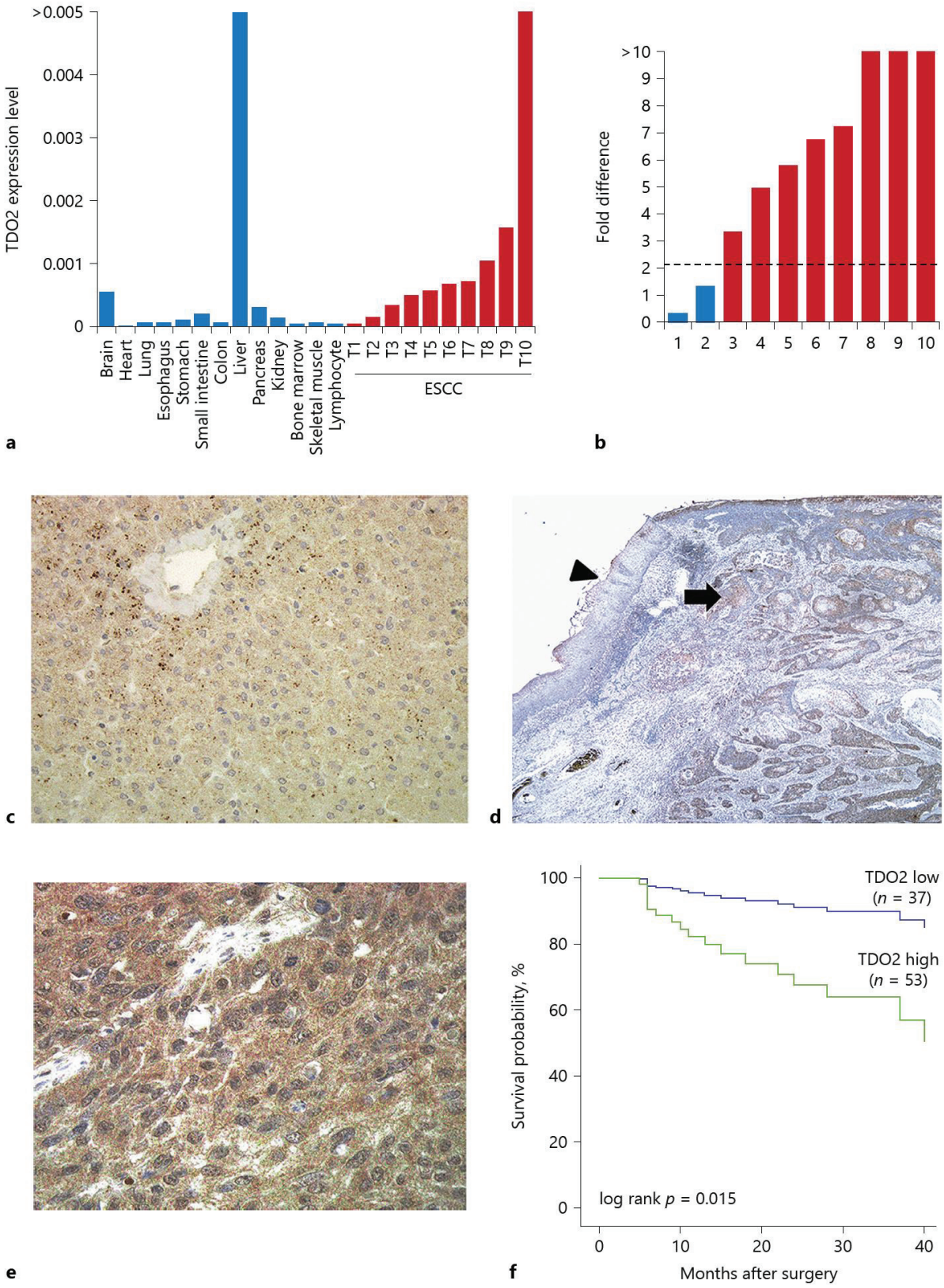

TDO2 expression level
>0.005
0.004
0.003
0.002
0.001
0
Brain
Heart
Lung
Esophagus
Stomach
Small intestine
Colon
Liver
Pancreas
Kidney
Bone marrow
Skeletal muscle
Lymphocyte
T1
T2
T3
T4
T5
T6
T7
T8
T9
T10
ESCC
a
Fold difference
>10
9
8
7
6
5
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10
b
c
d
e
Survival probability, %
100
80
60
40
20
0
TDO2 low
(n = 37)
TDO2 high
(n = 53)
log rank p = 0.015
0 10 20 30 40
Months after surgery
f

Fig.2

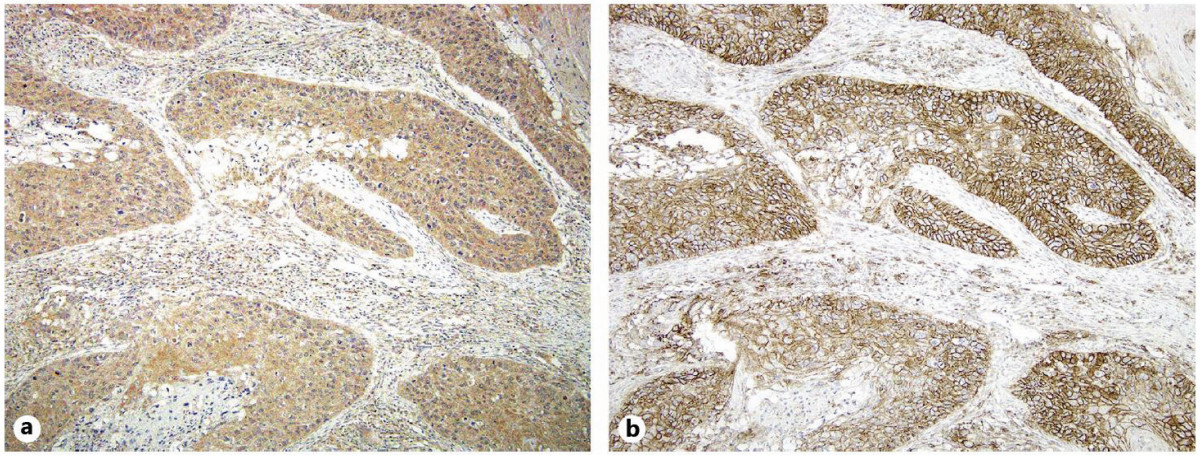


Fig.3

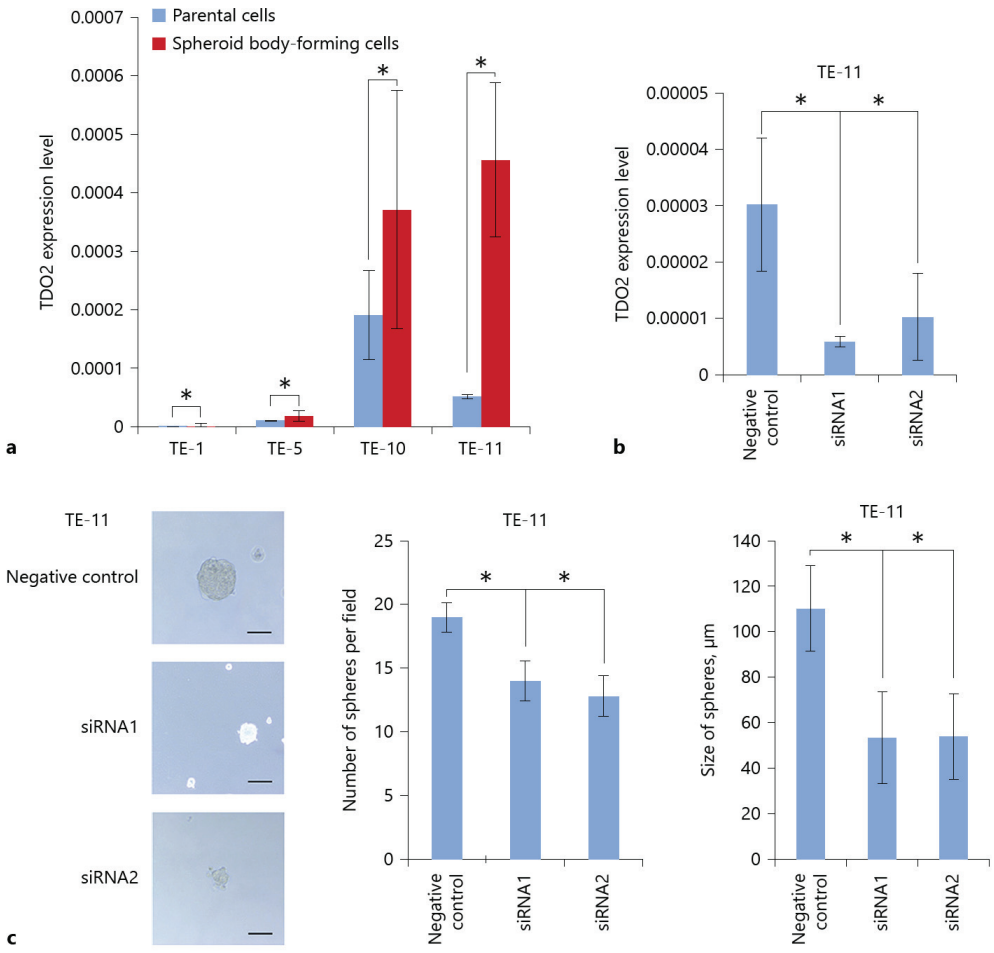
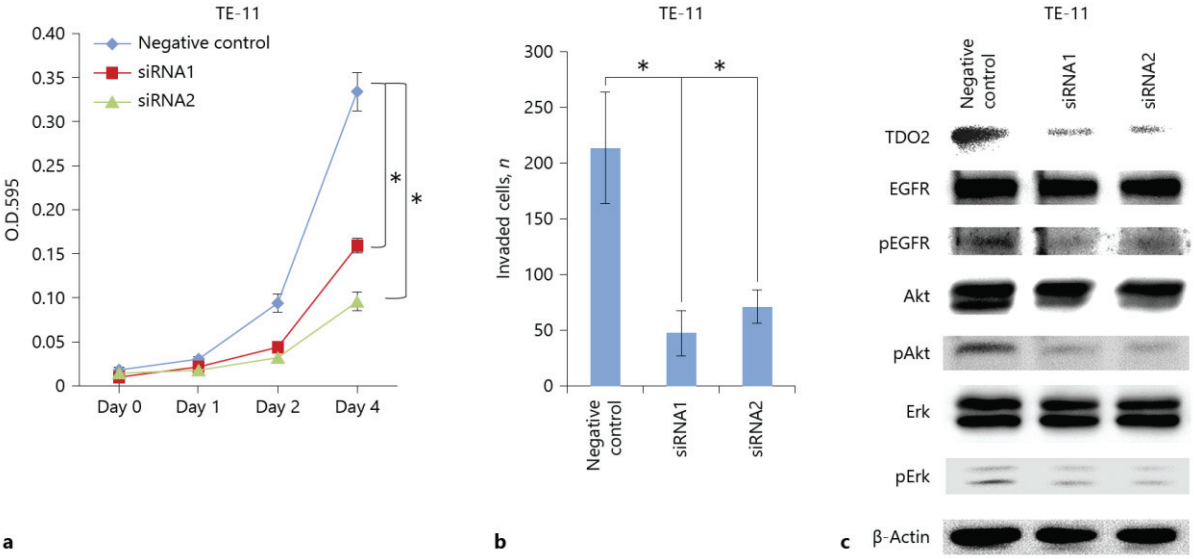
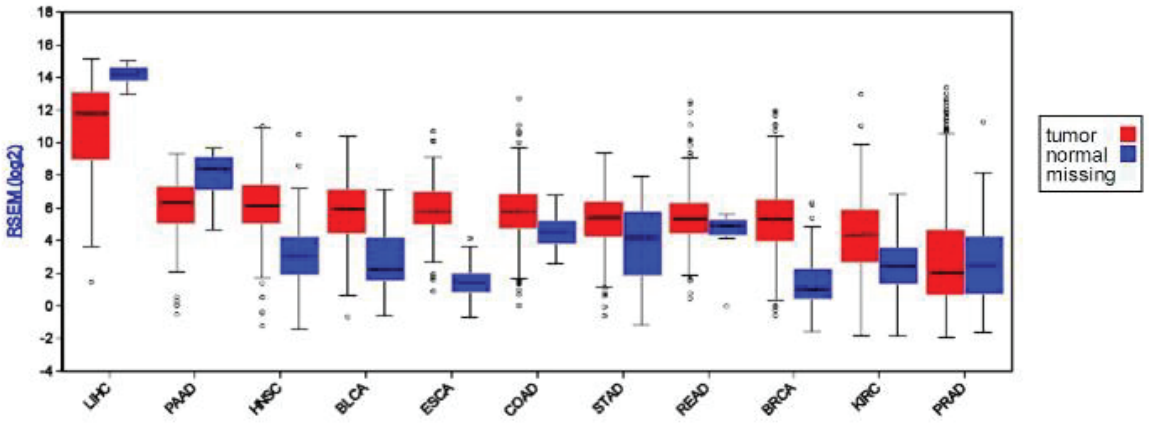

a
Parental cells
Spheroid body-forming cells
TDO2 expression level
0.0007
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0
TE-1
TE-5
TE-10
TE-11
b
TE-11
TDO2 expression level
0.00005
0.00004
0.00003
0.00002
0.00001
0
Negative control
siRNA1
siRNA2
c
TE-11
Negative control
siRNA1
siRNA2
TE-11
Number of spheres per field
25
20
15
10
5
0
TE-11
Size of spheres, μm
140
120
100
80
60
40
20
0

Fig.4

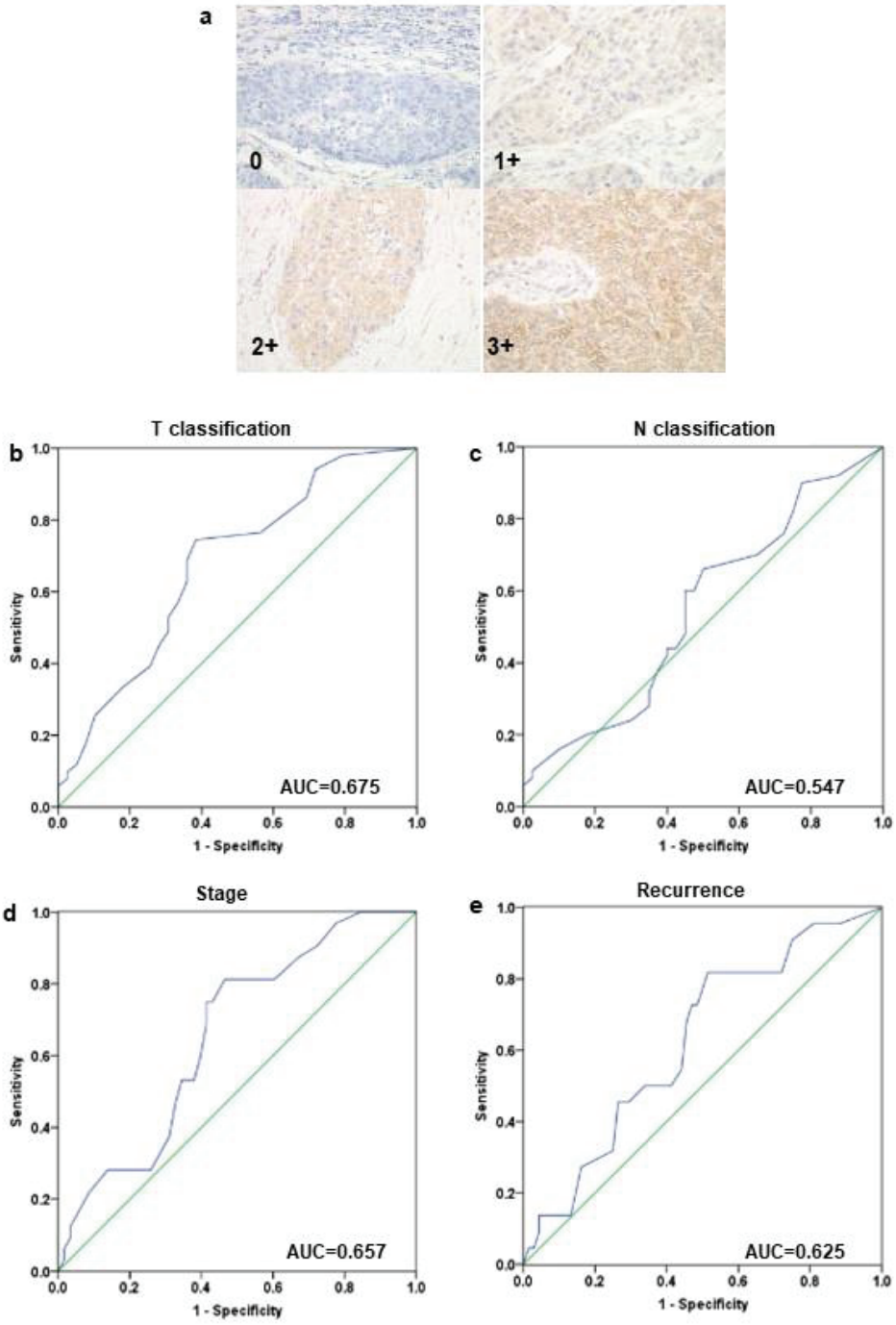

TE-11
Negative control
siRNA1
siRNA2
O.D.595
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
Day 0
Day 1
Day 2
Day 4
*
*
a
TE-11
Invaded cells, n
300
250
200
150
100
50
0
*
*
Negative control
siRNA1
siRNA2
b
TE-11
Negative control
siRNA1
siRNA2
TDO2
EGFR
pEGFR
Akt
pAkt
Erk
pErk
β-Actin
c

Supplementary Figure 1



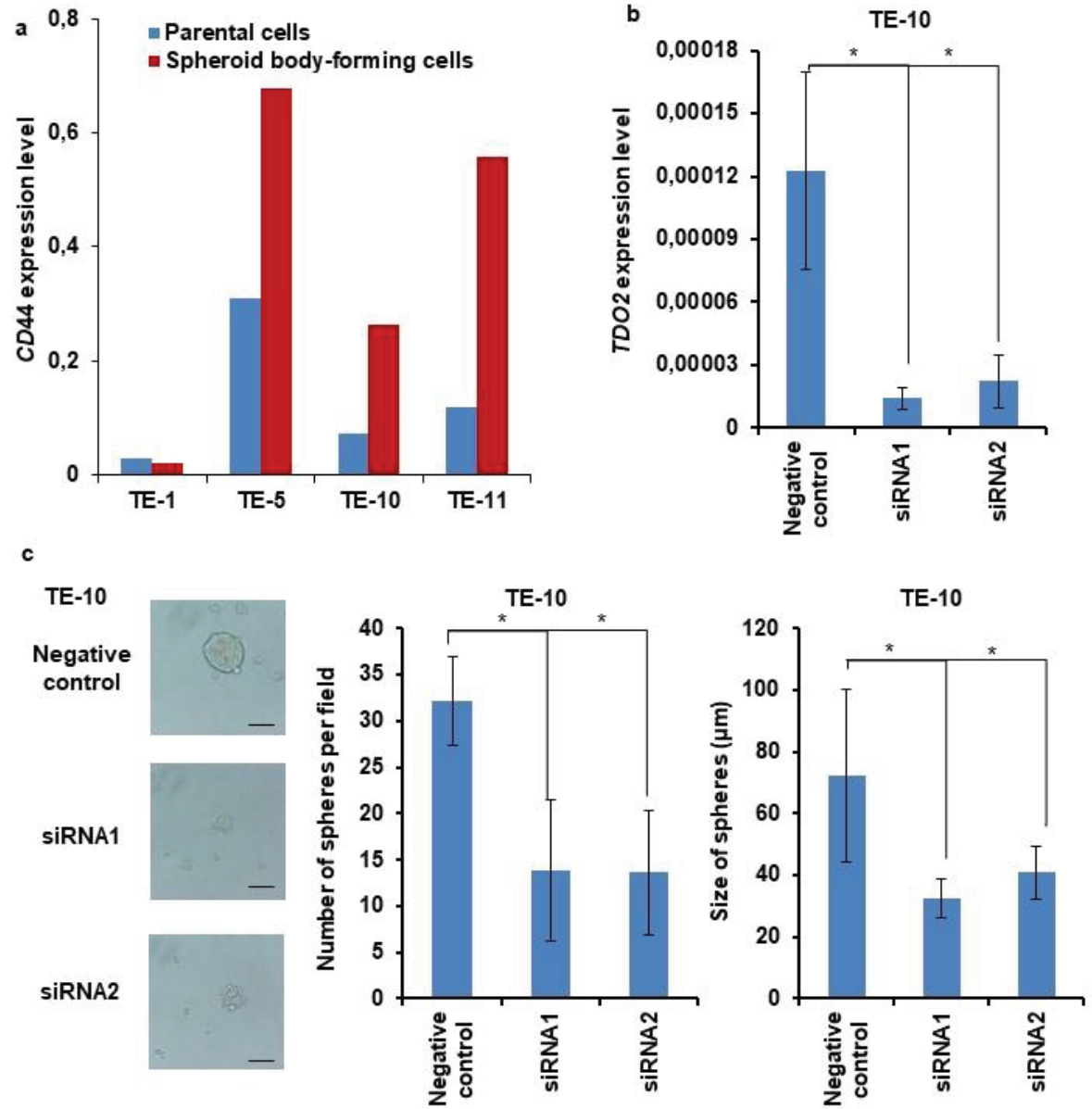
Supplementary Figure 1. The expression of TDO2 in various types of cancer from the TCGA dataset. LIHC: liver hepatocellular carcinoma; PAAD: pancreatic adenocarcinoma; HNSC: head and neck squamous cell carcinoma; BLCA: bladder urothelial carcinoma; ESCA: esophageal carcinoma; COAD: colon adenocarcinoma; STAD: stomach adenocarcinoma; READ: rectum adenocarcinoma; BRCA: breast invasive carcinoma; KIRC: kidney renal clear cell carcinoma; PRAD: prostate adenocarcinoma

Supplementary Figure 2



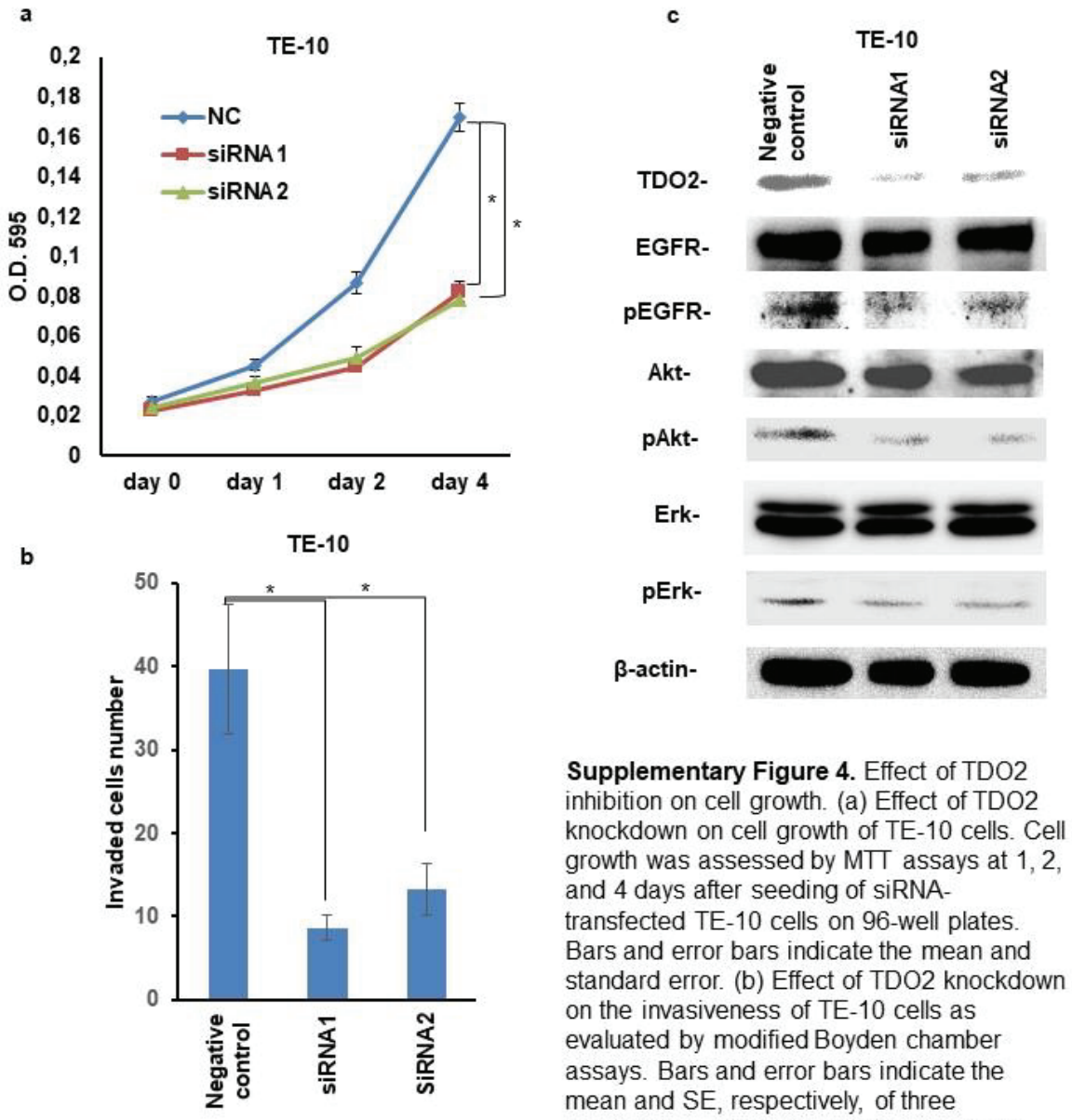
Supplementary Figure 2. Immunohistochemical analysis of TDO2 expression. (a) Immunoreactivity of TDO2 in tumor cells scored as 0 (no immunoreactivity) to 3+ (intense). Receiver operating characteristic curves for TDO2 expression score and (b) T classification, (c) N classification, (d) stage, and (e) recurrence. AUC, area under the curve.

Supplementary Figure 3



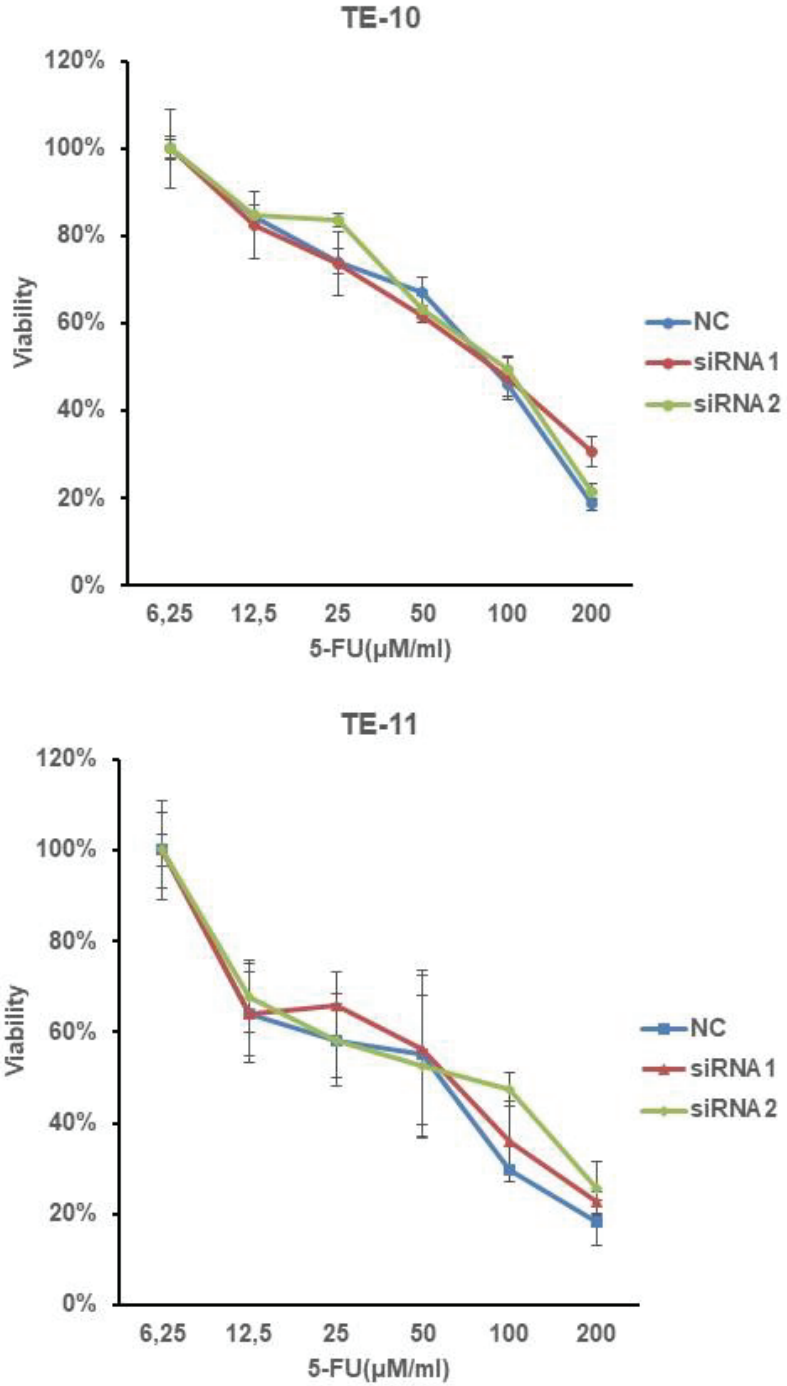
Supplementary Figure 3. Effect of TDO2 inhibition on spheroid formation. (a) CD44 mRNA is upregulated in spheroid body-forming cells compared with the parental cells in ESCC cell lines. (b) TDO2 mRNA expression was suppressed by siRNA transfection in TE10 cells. (c) The number and size of spheres from TE-10 cells transfected with the TDO2 siRNA or negative control siRNA. Bars and error bars indicate mean ± SD, respectively, of three different experiments. Black scale bar, 100 μm. *$p < 0.05$.

Supplementary Figure 4



Supplementary Figure 4. Effect of TDO2 inhibition on cell growth. (a) Effect of TDO2 knockdown on cell growth of TE-10 cells. Cell growth was assessed by MTT assays at 1, 2, and 4 days after seeding of siRNA-transfected TE-10 cells on 96-well plates. Bars and error bars indicate the mean and standard error. (b) Effect of TDO2 knockdown on the invasiveness of TE-10 cells as evaluated by modified Boyden chamber assays. Bars and error bars indicate the mean and SE, respectively, of three experiments. (c) Western blot analysis of TDO2 , epidermal growth factor receptor (EGFR), phospho-EGFR (pEGFR), Erk1/2, phospho-Erk1/2 (pErk1/2), Akt, and phospho-Akt (pAkt) in cell lysates from TDO2 cells transfected with TDO2 siRNA or negative control siRNA. β-Actin was included as a loading control. *$p < 0.05$.

Supplementary Figure 5



Supplementary Figure 5. Effect of TDO2 inhibition on drug resistance. Effect of TDO2 knockdown on drug resistance of TE-10 and TE-11 cells. Cell viability was assessed by MTT assays at 48 h after seeding of siRNA-transfected TE-10 cells on 96-well plates. Bars and error bars indicate the mean and standard error.