論　文　内　容　要　旨

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）

Oncology, 95(5):297-308, 2018.

主指導教員：安井　弥教授
（医系科学研究科　分子病理学）
副指導教員：武島　幸男教授
（医系科学研究科　病理学）
副指導教員：大上　直秀　准教授
（医系科学研究科　分子病理学）

PHAM QUOC THANG

（医歯薬保健学研究科　医歯薬学専攻）

Esophageal cancer is one of the deadliest cancers in the world. 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. Targeted therapies for esophageal cancer treatment currently remain limited. Although several clinical trials for targeted treatments of esophageal cancer have launched, only one study has enrolled patients with ESCC. Therefore, it is urgent to identify new biomarkers and develop a novel therapeutic target for esophageal cancer. Spheroid colony formation assays, an *in vitro?* technique of plating a limited number cells in culture dishes specifically coated for non-attachment in serum-free media, have been used to investigate cancer stem cell (CSC) characteristics. We previously analyzed the gene expression profile of spheroid colonies and parental cells derived from gastric cancer (GC) cell lines by microarray analysis, and identified several genes upregulated in spheroid colonies. Among those, tryptophan 2,3-dioxygenase (TDO2) is dramatically upregulated in MKN-1 cells (derived from adenosquamous cell carcinoma) compared with other GC cell lines. Overexpression of TDO2, an enzyme involved in tryptophan catabolism, promoted tumor cell survival and was correlated with tumor grade and poor prognosis in triple negative breast cancer and in brain tumors. However, the expression and biological significance of TDO2 in ESCC have not been investigated. In this study, we analyzed the expression 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, cell proliferation and invasion.

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/. For quantitative reverse transcription-polymerase chain reaction (qRT-PCR), we used 10 ESCC samples (tumor tissues and the corresponding non-neoplastic tissue). TDO2 protein expression was evaluated in 90 ESCC tissue samples by immunohistochemistry. Four ESCC cell lines (TE-1, TE-5, TE-10 and TE-11) were used to investigated biofunction of TDO2. TDO2 function in ESCC cell lines and spheroid colony formation was evaluated by using RNAi. Cell growth was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Modified Boyden chamber assays were performed to examine cell invasiveness.

Results: In the TCGA datasets, TDO2 expression was upregulated in most cancers except for liver cancer and pancreas cancer. The fold change expression of TDO2 between normal and cancer tissue was the highest in esophageal cancer. Next, the

expression of TDO2 was evaluated 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 highest in liver. TDO2 mRNA levels in ESCC tissue samples were higher than those in normal tissues. Calculation of the T/N ratios for the ESCC cases by qRT-PCR demonstrated that expression of TDO2 was upregulated in 8 out of the 10 ESCC cases (80%). Immunohistochemical analysis using 90 ESCC tissue samples showed that TDO2 high expression was associated with advanced T classification ($p=0.001$, tumor stage ($p=0.001$) and recurrence status (0.012). Kaplan-Meier analysis demonstrated that TDO2 high expression ESCC cases showed poorer survival than TDO2 low expression ESCC cases ($p=0.015$). Furthermore, ESCCs with high TDO2 expression showed significantly enriched numbers of CD44-positive cells ($p=0.026$).

The effect of TDO2 inhibition by siRNA transfection on sphere number and size was examined. qRT-PCR revealed that spheroid body-forming cells showed enriched CD44 expression. TDO2 mRNA levels were also dramatically upregulated in spheroid body-forming cells compared with the parental cells in all four ESCC cell lines. TE-10 and TE-11 cells, which exhibited high levels of TDO2 mRNA expression in spheroid body-forming and parental cells were selected for further analysis. TE-11 and TE-10 cells transfected with TDO2 siRNA showed reduced number and size of spheres compared with negative control transfected cells. 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. EGFR activates the RAS-MEK-ERK and AKT-PI3K pathways, leading to cancer cell proliferation and survival. The effect of TDO2 inhibition on the EGFR signaling pathway was studied. Western blot analysis confirmed successful TDO2 knockdown in TE-11 and TE-10 cells transfected with TDO2 siRNA. The levels of phosphorylated EGFR, Erk and Akt were lower in TDO2 siRNA1- and siRNA2-transfected TE-11 and TE-10 cells compared with negative control siRNA-transfected cells.

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.