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学 位 論 文

CD40 Expression in Human Esophageal Squamous Cell
Carcinoma Is Associated with Tumor Progression and
Lymph Node Metastasis

(食道扁平上皮癌における CD40 の発現は腫瘍の進行とリンパ節転移に関する)

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CD40 Expression in Human Esophageal Squamous Cell Carcinoma Is Associated with Tumor Progression and Lymph Node Metastasis

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Abstract. *Background: The co-stimulatory molecule cluster of differentiation 40 (CD40) is widely expressed in various types of malignant tumors, but its role remains unclear. The purpose of this study was to investigate the relationship between CD40 expression and clinicopathological variables in patients with esophageal squamous cell carcinoma (ESCC), as well as the function of CD40 expressed on ESCC tumor cells in vitro. Materials and Methods: Tumor specimens of patients who underwent surgical resection for ESCC were immunohistochemically analyzed for CD40 expression. Results: Of the 122 specimens, 45 (37%) were positive for CD40. Significant positive correlation was found between CD40 expression and p-stage ($p=0.0011$), histopathological grade ($p=0.0143$), pT-classification ($p=0.0011$), and pN-classification ($p=0.0007$). Survival of patients with stage III and IV disease with positive CD40 expression was significantly shorter than that of those with negative expression (log-rank test, $p=0.0422$). In in vitro analysis, while the addition of recombinant human CD154 did not inhibit growth, it did induce a significant increase in interleukin 6 production in ESCC cell lines. Conclusion: These results suggest that functional expression of CD40 on tumor cells might play an important role in tumor progression and lymph node metastasis in ESCC.*

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Key Words: CD40, CD154, CD40 ligand, CD40L, esophageal squamous cell carcinoma, IL6.

Esophageal cancer is the sixth most common cause of cancer deaths in males, with an estimated 455,800 new esophageal cancer cases and 400,200 deaths worldwide reported in 2012 (1). While the number of reported cases in the United States has declined in recent years, the incidence of esophageal cancer is now highest in Eastern Asia and in Eastern and Southern Africa, with 90% of these cases identified as squamous cell carcinomas (2). Despite significant progress in recent years in preoperative treatments in the neoadjuvant setting followed by radical surgical resection as a standard therapeutic approach for locally advanced esophageal cancer (3, 4), prognosis remains poor. Further improvements in survival require new biological parameters that can be used to develop novel therapeutic strategies for esophageal cancer. The co-stimulatory molecule cluster of differentiation 40 (CD40), a member of the tumor necrosis factor receptor superfamily, is predominantly expressed on antigen-presenting cells including B-cells, dendritic cells, and macrophages (5, 6). Its ligand CD154 (or CD40L), a 39-kDa membrane glycoprotein, is primarily expressed on activated T-cells. Binding of CD40 to its ligand induces activation and differentiation of B-cells (7, 8), the production of various cytokines, and up-regulation of adhesion and co-stimulatory molecules (intercellular adhesion molecule 1, vascular cell adhesion molecule 1, CD80, and CD86) (6, 9, 10) and plays a crucial role in both humoral and cellular immunity (5, 6).

CD40 is also widely expressed on various types of malignant tumors, such as melanoma (11, 12), ovarian cancer (13), breast cancer (14, 15), non-small cell lung cancer (16, 17), colon cancer (18), gastric cancer (19), bladder cancer (20), prostate cancer (21), and renal cell carcinoma (22-25). However, the role of CD40 on tumor cells in malignant progression and in the cancer microenvironment remains unclear. In melanoma (12), bladder (20), gastric (19), and non-small cell lung (16, 17) cancer, CD40 expression was demonstrated to be associated with tumor progression and

poor prognosis. However, the expression of CD40 in esophageal squamous cell carcinoma (ESCC) and its function have not been demonstrated. The purpose of this study was, therefore, to evaluate the expression of CD40 in a cohort of patients with ESCC and to investigate the biological function of CD40 expressed on ESCC cells *in vitro*.

Materials and Methods

Cell lines and peripheral blood mononuclear cell isolation. The human esophageal squamous cell carcinoma cell lines TE2, TE4, TE5, TE8, TE9, TE10, TE13, and TE14 were obtained from the Japanese Cell Resource Center for Biomedical Research (Sendai, Japan). HEC46 was provided by Dr. T. Toge (University of Hiroshima, Japan), and SGF7 was provided by Dr. T. Saito (Toyama Medical and Pharmaceutical University, Japan). All cell lines were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Yokohama, Japan) and 1% penicillin-streptomycin, and were maintained in a humidified atmosphere with 5% CO₂. Peripheral venous blood samples were obtained from healthy adult donors. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by density gradient centrifugation on Ficoll-paque Plus (GE Healthcare, Uppsala, Sweden), according to the manufacturer's instructions. After centrifugation of the blood samples, the PBMC layer was carefully transferred and the cells were washed three times with phosphate-buffered saline (PBS).

Western blot analysis. Western blot analysis was performed to confirm CD40 expression in ESCC cell lines. Twenty micrograms of cell lysate from each sample was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% SDS-polyacrylamide gels, and the protein was transferred to polyvinylidene difluoride membranes (Amersham, Aylesbury, UK). For western blotting, mouse anti-CD40 monoclonal antibody (diluted 1:250; NOVOCASTRA, Newcastle, UK) was used as the primary antibody and the appropriate peroxidase-conjugated goat anti-mouse IgG antibody (1:10,000; Jackson Immuno Research Laboratories, West Grove, PA, USA) was used as the secondary antibody. Chemiluminescent detection of bound antibody was carried out using the ECL system (Amersham). Mouse anti-actin antibody (1:250; EMD Millipore, Darmstadt, Germany) was also used as an internal control. Lysates of normal human PBMCs were used as a positive control for CD40 and lysates of normal human esophageal tissue were used as a negative control.

Cell proliferation assay. Cell proliferation was determined using a tetrazolium dye conversion [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8)] assay (Cell Counting Kit-8; Wako Chemicals, Osaka, Japan). In order to assess cell viability, quadruplicate wells containing 5×10³ cells per well were cultured in 96-well plates with or without interferon-gamma (IFN γ ; R&D systems Inc., Minneapolis, MN, USA) at a final concentration of 100 ng/ml for 24 hours, and recombinant human soluble CD154 (ALX-582-054-K101; Alexis, Plymouth Meeting, PA, USA) was added at a final concentration of 1000 ng/ml followed by culture for an additional 48 hours. Dye conversion was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices,

Tokyo, Japan) at wavelengths of 450 nm and 600 nm after 2 h incubation at 37°C. Cell proliferation was determined by calculation of the absorbance of viable cells as measured against control wells containing PBS alone.

ELISA measurement of IL6. To measure IL6 levels in cell culture supernatants, triplicate wells containing TE10, HEC46, or SGF7 cells (2×10⁵ cells per well) were cultured in 24-well plates with IFN γ (100 ng/ml) for 24 h and the cells were then stimulated with PBS or recombinant human soluble CD154 (rhCD154) at a final concentration of 1,000 ng/ml. After additional culture for 48 h, the supernatants were harvested and the concentration of IL6 was measured using the commercially available Human IL-6 Quantikine Enzyme Linked Immunosorbent assay (ELISA) kit (R&D Systems Inc.), according to the manufacturer's instructions. The final concentration of IL6 was normalized to the cell number.

Flow cytometric analysis of surface CD40 expression. The expression of CD40 on the surface of ESCC cells was examined using flow cytometric analysis. Briefly, 4×10⁵ cells were seeded into 12-well plates and cultured with or without recombinant human IFN γ (100 ng/ml) for 48 h. The cells were then trypsinized with 1 ml of 0.25% trypsin for 10 minutes, washed with PBS, and resuspended in 100 μ l of PBS supplemented with 2% fetal bovine serum. The cell suspension was incubated on ice for 30 min with a fluorescein isothiocyanate (FITC)-conjugated anti-CD40 monoclonal antibody (555588; BD Biosciences, San Jose, CA, USA). In parallel, FITC-conjugated mouse IgG1k (554679; BD Biosciences) served as an isotype control. The expression of CD40 was then analyzed using CellQuest software (BD Biosciences). PBMCs were included as a positive control.

Patients and specimens. This study was conducted at the Hokkaido University in accordance with Institutional Review Board guidelines. One hundred and twenty-two patients (105 men and 17 women; mean age, 62.3 years) with primary ESCC underwent radical esophagectomy between September 1989 and May 1999 at the Department of Surgical Oncology, School of Medicine, Hokkaido University, or at two affiliated hospitals. No distant metastasis was detected in any patient upon preoperative examination. No patient had received prior anticancer treatment. Cases of in-hospital death were excluded from the current study. The clinical typing of tumors was determined according to the TNM classification system of the International Union Against Cancer (26). All specimens were fixed in 10% formalin and embedded in paraffin wax. One of the deepest sections from each tumor was selected for evaluation, and serial 4- μ m-thick sections were examined using immunohistochemistry.

Immunohistochemical examination. Immunohistochemical reactions were performed using the universal immuno-enzyme polymer method. Sections were deparaffinized in xylene, dehydrated through a graded ethanol series, and placed in a plastic container containing EDTA buffer (pH 8.0). After heating in a pressure cooker for 2 min at the highest pressure, the sections were cooled and endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol for 10 minutes. The sections were washed three times in PBS containing 1% Tween 20, followed by incubation in 10% normal goat serum (Nichirei Cooperation, Tokyo, Japan) for 30 min. Anti-human CD40 mouse monoclonal antibody (11E9;

Novocastra, Newcastle, UK), was applied at 1:40 dilution and specimens were then incubated individually overnight at 4°C. After three additional washes with PBS, sections were incubated with a biotinylated goat antibody to mouse immunoglobulin [Histofine Simple Stain MAX-PO (MULTI); Nichirei Cooperation] for 30 min at room temperature. Sections were washed with PBS, and the immunohistochemical reactions were visualized by incubation for approximately 5 min with 3-3'-diaminobenzidine tetrahydrochloride (Histofine Simple Stain DAB Solution; Nichirei Cooperation), followed by washing in distilled water. Sections were counterstained in hematoxylin for 1 minute and were then mounted in Permount (Micro Slide; Muto-Glass, Tokyo, Japan). Normal human lymph nodes were used as a positive tissue control for CD40. The negative reagent control for CD40 consisted of serial tissue sections of each sample in which isotype-matched negative control mouse IgG (IgG2b; X0944, Dako, Tokyo, Japan) was used as a primary antibody.

Classification of CD40 staining in ESCC. We selected sections that contained the deepest site for each tumor, and these immunostained sections were evaluated under a microscope (Olympus Optical Co., Ltd., Tokyo, Japan). The degree of staining was counted in more than 10 independent high-power microscopic fields (×400) for each tissue sample. The five areas containing the highest density of reactive cells were evaluated in each case, and the mean percentage of cancer cells with positive staining for CD40 was determined. Tissue sections with fewer than four reactive tumor cells per high power field (HPF) were considered CD40-negative and those with four or more reactive cells HPF were defined as CD40-positive. Three investigators of our study (Y.M., K.I., and T.I.) independently evaluated the immunohistochemical labeling of all specimens, without knowledge of patient background or outcome. Immunoreactivity in each section was represented by the median scores evaluated by the three independent investigators.

Statistical analysis. For correlations between CD40 status and patients' clinicopathological variables, statistical significance was evaluated by the χ^2 test and Fisher's exact test. For the IL6 ELISA, the comparison of two groups was performed using Student's *t*-test. The cumulative survival rate was calculated using the Kaplan-Meier method. Statistical significance was analyzed by the log-rank test. Overall survival rates were calculated using the period from surgery to death. Probability values of less than 0.05 were considered statistically significant in all of the analyses. All analyses were performed with statistical software (Prism 6; Graphpad, La Jolla, CA, USA).

Results

CD40 expression in human ESCC. CD40 expression was immunohistochemically analyzed in 122 ESCC specimens. Representative photomicrographs of positive and negative immunohistochemical staining for CD40 are shown in Figure 1. Each specimen contained lymphocytes that stained for CD40, which served as internal positive controls (Figure 1A). Within the tumor, the distribution of CD40 staining was heterogeneous, and CD40 immunoreactivity was detected both in the cytoplasm and on the plasma membranes of cancer cells (Figure 1B); no reactivity was observed in normal esophageal mucosa (Figure 1C). For the microscopic

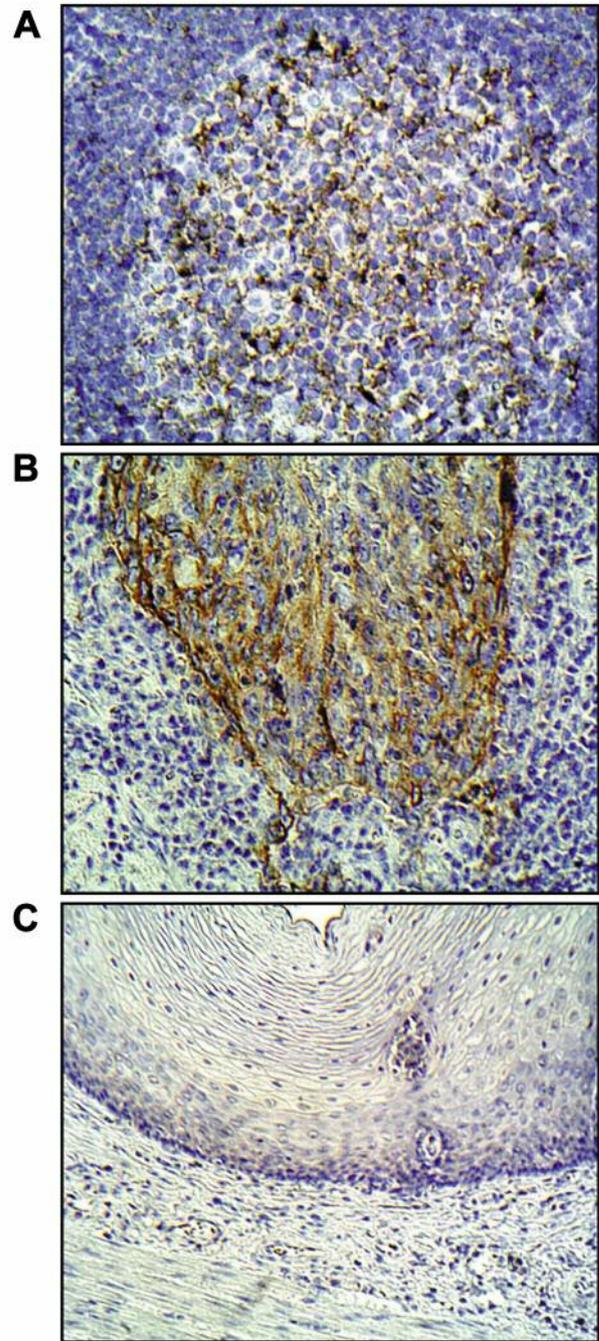


Figure 1. Representative immunohistochemical staining of surgical specimens for CD40. A: Positive expression of CD40 in a normal lymph node served as a positive control. B: Positive expression of CD40 in the cell membranes and cytoplasm of esophageal squamous cell carcinoma cells (original magnification, ×400). C: Negative expression of CD40 in normal esophageal mucosa. Original magnification, ×200.

classification of CD40 expression on tumor cells, the morphological findings of each cell were carefully evaluated to minimize the risk of including CD40-positive monocytes,

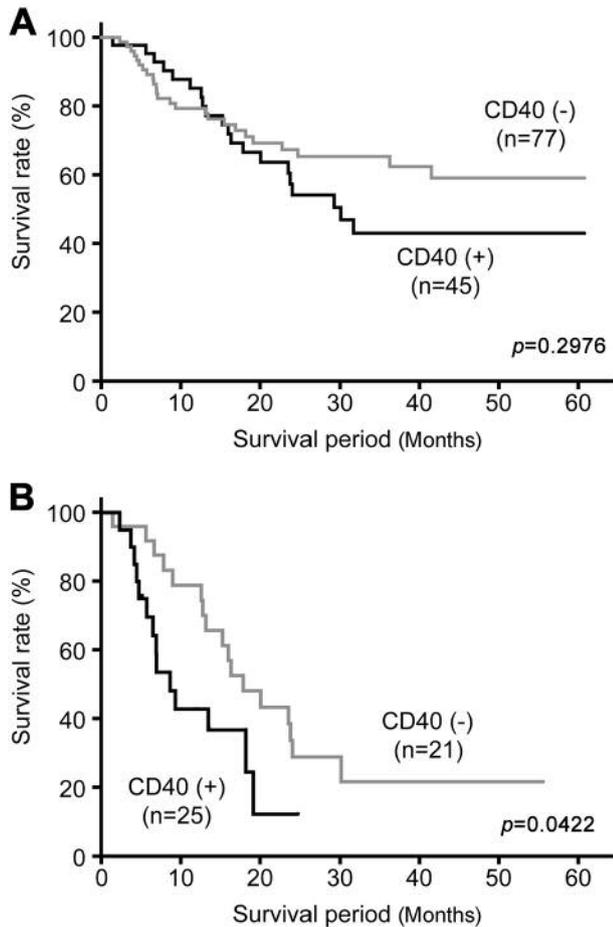


Figure 2. Kaplan-Meier analysis for overall survival according to CD40 expression in patients with all stages of esophageal squamous cell carcinoma (ESCC) (A) in patients with stage III and IV ESCC (B).

as previously described (17, 25, 27). Based on this analysis, CD40 expression was defined as positive in 45 cases (36.9%) and negative in 77 cases (63.1%).

Correlation between CD40 status and clinicopathological variables. Correlations between the status of CD40 expression and clinicopathological features in ESCC are summarized in Table I. There were no significant correlations with age, gender, or M-classification. CD40 status was significantly positively correlated with pStage ($p=0.0011$), histopathological grade ($p=0.0143$), pT-classification ($p=0.0011$), and pN-classification ($p=0.0007$).

Kaplan–Meier survival analysis. Survival curves according to CD40 expression were constructed according to the Kaplan–Meier method (Figure 2). Among 122 patients with ESCC, the survival rates for patients positive for CD40 expression were lower than for those negative for CD40

Table I. Association between CD40 expression and clinicopathological variables in patients with esophageal squamous cell carcinoma.

Variables	Number of cases	CD40		p-Value
		Positive (n=45)	Negative (n=77)	
Gender				
Male	105	42	63	0.1045
Female	17	3	14	
Age, years				
≥60	79	34	45	0.0768
<60	43	11	32	
pStage				
I	38	5	33	0.0011*
II	38	15	23	
III	27	16	11	
IV	19	9	10	
Histopathological grade				
G1	32	5	27	0.0143*
G2	62	27	35	
G3	28	13	15	
pT classification				
T1	54	10	44	0.0011*
T2	13	7	6	
T3	43	24	19	
T4	12	4	8	
pN classification				
N1	60	13	47	0.0007*
N2	62	32	30	
pM classification				
M0	101	35	66	0.3220
M1	21	10	11	

*Significantly different.

expression, but the difference was not statistically significant (log-rank test, $p=0.2976$; Figure 2A). In selected cases with stage III and IV disease ($n=46$), the survival rates for patients positive for CD40 expression were significantly lower than for those negative for CD40 expression (log-rank test, $p=0.0422$; Figure 2B), suggesting that CD40 expression might be associated with poor prognosis of patients with advanced ESCC.

CD40 expression in human ESCC cell lines. In order to examine the biological function of CD40 expressed in ESCC cells, we first evaluated ESCC cell lines for CD40 expression *in vitro*. Of the 11 ESCC cell lines tested, TE5, TE10, and HEC46 were positive for CD40 expression (>1.0%) by flow-cytometric analysis (Figure 3A). This finding was confirmed by western blot analysis in which TE5, TE10, and HEC46 showed high levels of a 42-kDa protein that cross-reacted with the anti-CD40 antibody

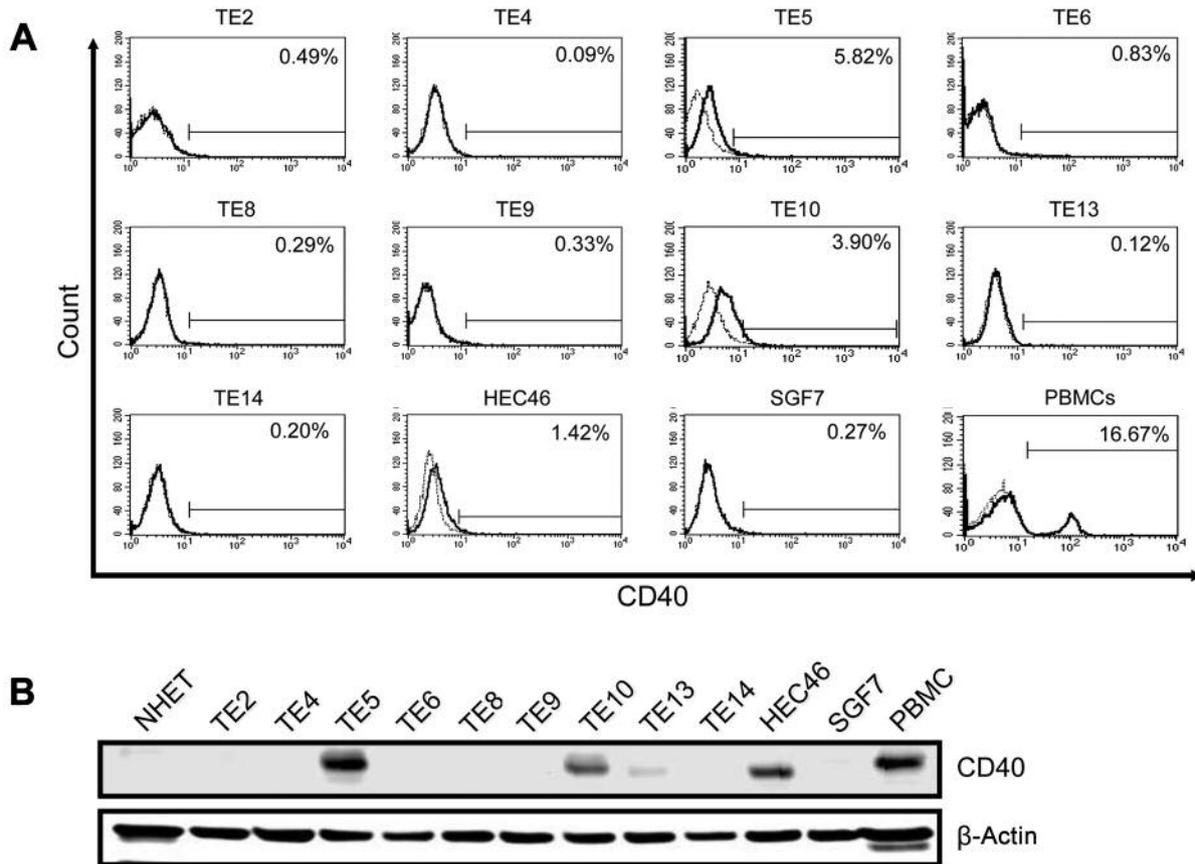


Figure 3. CD40 expression in esophageal squamous cell carcinoma (ESCC) cell lines. A: The level of cell surface CD40 expression on each ESCC cell line was analyzed using fluorescein isothiocyanate conjugated anti-CD40 monoclonal antibody (black) and flow cytometry. Background staining is indicated in grey. B: Western blots of cell lysates were probed with an anti-human CD40 antibody, or anti- β -actin antibody as an internal control. A lysate from normal human peripheral blood mononuclear cells (PBMC) was used as a positive control and a lysate from normal human esophageal tissue (NHET) was used as a negative control.

compared to the other ESCC cell lines (Figure 3B). In addition, treatment of CD40-positive cell lines with IFN γ resulted in an increase in cell-surface CD40 expression as assessed by flow-cytometric analysis (Figure 4).

Effects of recombinant CD154 on ESCC cells in vitro. As CD40 interacts with CD154 to regulate its signaling pathways, *in vitro* proliferation was then examined by the WST-8 assay following exposure of two CD40-positive (TE10, HEC46) and one CD40-negative (SGF7) ESCC cell lines to rhCD154 with and without IFN γ stimulation (Figure 5). IFN γ stimulation resulted in significant growth suppression of the CD40-positive cell lines, however, rhCD154 stimulation had no significant growth-inhibitory effect on any cell line.

In order to evaluate the association between CD40 signaling and cytokine production in ESCC, the culture supernatants from cells were examined for IL6 by ELISA

(Figure 6). IL6 production was significantly increased in all three cell lines following IFN γ stimulation. Furthermore, an additive increase in IL6 secretion was observed when cells were treated with rhCD154 in combination with IFN γ , even in SGF7 cell line with low CD40 expression.

Discussion

In the present study, out of the 122 surgically resected ESCC, 45 cases (37%) were classified as positive for CD40 expression. This incidence of CD40 expression was similar to that reported for other cancer types. Previous immunohistochemical studies reported CD40 expression in 69.2% of pancreatic adenocarcinomas (28), and in 51.9% of non-small cell lung cancer cases (17). We also found that patients with tumors that were classified as positive for CD40 expression had a more advanced pathological stage, poorer differentiation, higher frequency of positive lymph

node metastasis, and a more extensive tumor. These correlations suggest that CD40 expression in the tumor itself might up-regulate tumor progression and metastatic potential in ESCC. In clinical analysis of human lung cancer, it has been reported that positive CD40 expression in tumors showed a significant correlation with metastatic spread (12). By binding with its ligand CD154, CD40 expressed on the endothelial cell surface was shown to up-regulate the production of cytokines and chemokines, and also to increase the expression of co-stimulatory and adhesion molecules and the production of matrix metalloproteinases (6, 10, 29). Likewise, in ESCC, our results of activation of the CD40 pathway by CD154 or $IFN\gamma$, based on induction of IL6 production, suggest that these other factors may also be increased following stimulation and may allow cancer cells to spread to the regional lymph nodes.

The prognostic significance of CD40 expression in tumors depends on the cell type and remains controversial. In terms of survival, we found that patients with CD40-positive tumors had a shorter survival compared to patients with CD40-negative tumors, although the difference between them was not statistically significant. However when cases with stage III and IV disease were selected, the overall 5-year survival rate for patients with positive CD40 expression (12.2%) was significantly lower than for those with negative expression (21.7%). Our data are consistent with a number of previous reports of other tumor types. Thus, a correlation between CD40 expression on tumor cells and poor prognosis has been reported in melanoma (12), bladder cancer (20), gastric cancer (19), and lung cancer (16, 17). However, in contrast, positive CD40 expression was associated with better survival in patients with renal cell carcinoma (25). The reason for this discrepancy remains unclear.

In order to identify the role of the CD40 signaling pathway in ESCC, we evaluated whether CD40 signaling correlated with cancer cell growth of ESCC *in vitro*. In the ESCC cell lines tested, we found that CD40 stimulation mediated by rhCD154 ligation did not inhibit growth in a cell proliferation assay. This result is in contrast to previous reports of a direct anti-proliferative effect induced by CD40–CD154 interaction in various cancer cells *in vitro* or *in vivo* (30, 31). On the other hand, recent studies demonstrated that stimulation of CD40 protects human bladder carcinoma cells from apoptosis (32), and that a low level of CD40 expression on tumor cells was not associated with cell growth in head and neck squamous cell cancer and ovarian cancer (27, 33). Thus, the effect of CD40 on tumor proliferation differs depending on the cancer type, and in ESCC, a direct anti-proliferative effect of CD40 may not be evident due to the low level of CD40 expression in the ESCC cell lines (less than 20%).

In addition to exerting a direct anti-proliferative effect on tumor cells, previous studies have demonstrated a role for CD40 in immunosuppression within the tumor micro-

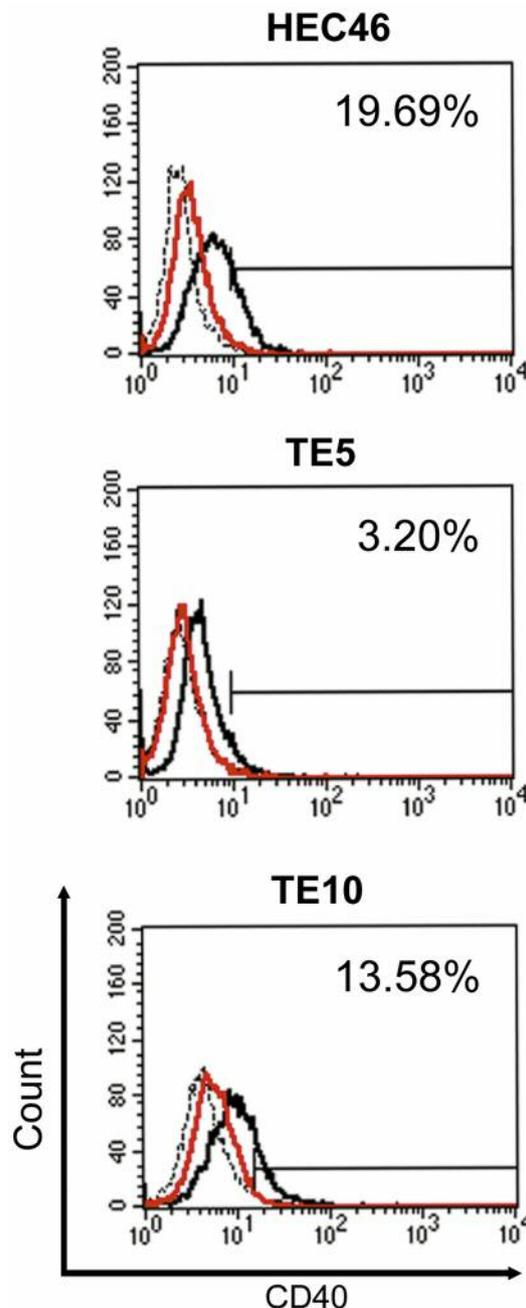


Figure 4. Interferon gamma ($IFN\gamma$) stimulation increased cell surface CD40 expression in esophageal squamous cell carcinoma cell lines. Cell surface CD40 was analyzed using flow cytometry of the indicated cell lines following treatment with (black line) or without (red line) $IFN\gamma$ (100 ng/ml). $IFN\gamma$ -treated cells stained with isotype control IgG are indicated with a broken line.

environment. In CD40-positive tumors, CD40 reportedly suppressed immune responses through down-regulation of CD154 and inactivated T-cell responses (34). Furthermore, direct interaction between the CD40 expressed on tumor cells

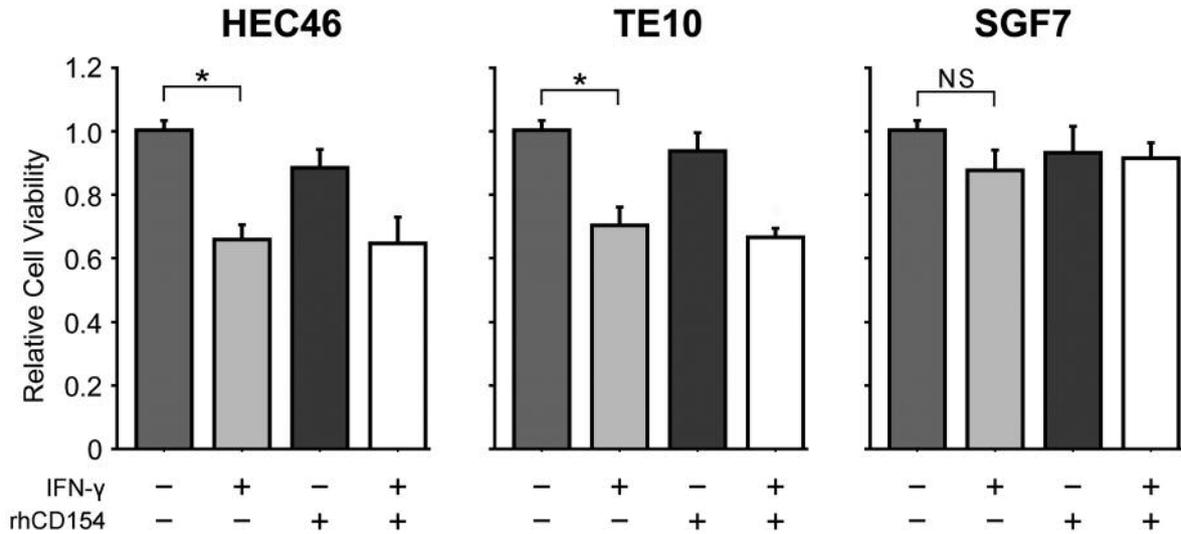


Figure 5. Effect of soluble recombinant human CD154 (rhCD154) treatment combined with interferon gamma (IFN γ) stimulation on esophageal squamous cell carcinoma cell proliferation. The proliferation of HEC46, TE10 and SGF7 cell lines was assessed following addition of IFN γ (100 ng/ml) on day 1 with/without rhCD154 (1,000 μ g/ml) on day 2 as indicated. Cell proliferation was determined by calculation of the absorbance of viable cells as measured against control wells containing phosphate buffered saline alone. * $p < 0.05$; NS: not significant, $p \geq 0.05$.

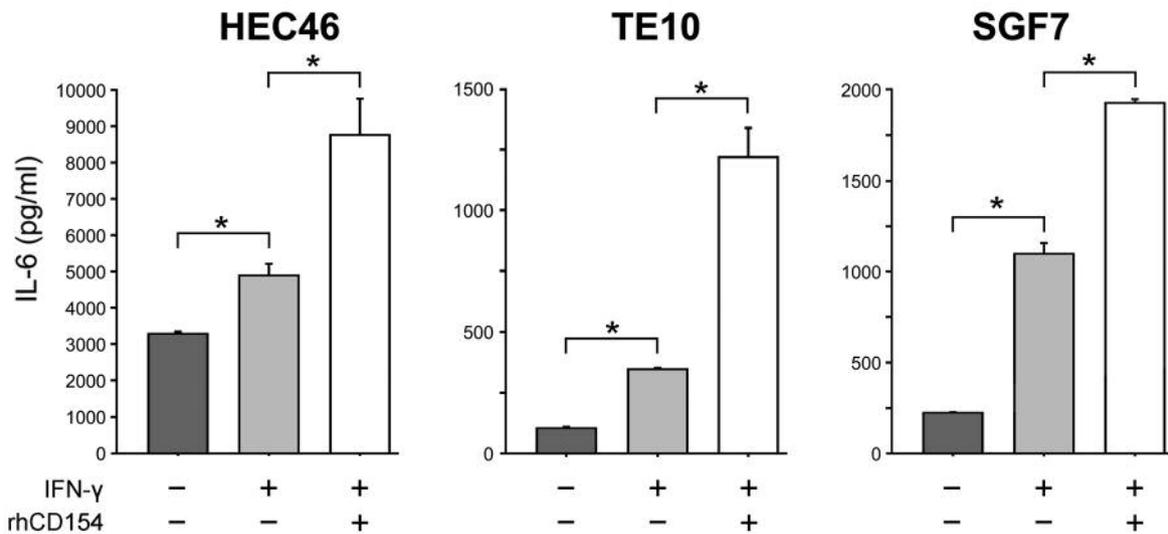


Figure 6. Interleukin 6 (IL6) secretion following stimulation of esophageal squamous cell carcinoma cell lines with recombinant human CD154 (rhCD154) with/without interferon gamma (IFN γ). * $p < 0.05$.

and CD154 on activated T-cells increased TGF β production and the differentiation of Th17 cells, resulting in acceleration of cancer cell proliferation (35). These findings strongly indicate that CD40 expression might work in favor of cancer progression and immuno-evasion mechanisms in the cancer microenvironment. In the present study, a significant increase in IL6 production was induced by rhCD154 in ESCC cell lines,

even in the cell line with low CD40 expression. Previous studies have demonstrated that CD40 induces the up-regulation of IL6 production in various tumors including multiple myeloma (36), breast cancer (35), ovarian carcinoma (33), and non-small cell lung cancer (37). Furthermore, IL6 has been reported to induce tumor progression in various human malignancies, including esophageal cancer (38). In ESCC, IL6

has been significantly associated with poor prognosis (39), and acts as an autocrine anti-apoptotic growth factor to support tumor progression (40). These previous findings regarding CD40 and IL6 support the idea that IL6 may play an important role as one of the central factors in CD40-mediated tumor progression in ESCC.

To our knowledge, this is the first study to show the expression of functional CD40 on ESCC cells, and our results suggest that the high incidence of CD40 expression is associated with tumor differentiation, extent of the tumor, and lymph node metastasis in ESCC. Combined with previous findings that show immunosuppressive functions of CD40 signaling in cancer progression, these data suggest that it may be possible to re-activate immunoresponsiveness against ESCC by using potential immunotherapeutic tools such as an anti-CD40 antagonistic antibody or by silencing CD40 expression.

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Disclosure Statement

All Authors declare no competing financial interests.

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