Apurinic/Apyrimidinic endonuclease-1 is associated with angiogenesis and VEGF production via upregulation of COX-2 expression in esophageal cancer tissues

Running title: APE-I is associated with angiogenesis and VEGF production

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Abstract

Objective Apurinic/apyrimidinic endonuclease-1 (APE-1) is a key enzyme responsible for DNA base excision repair (BER) and is also a multifunctional protein such as redox effector for several transcriptional factors. Our study was designed to investigate APE-1 expression and to study its interaction with COX-2 expression and VEGF production in the esophageal cancer.

Materials and Methods The expression of APE-1, COX-2, MCP-1, CCR2 and VEGF were evaluated by immunohistochemistry in 65 human esophageal squamous cell carcinoma (ESCC) tissues. Real-time PCR and western blotting were performed to detect mRNA and protein expression of APE-1 and p-STAT3 expression in MCP-1 stimulated ESCC cell lines (KYSE 220 and EC-GI-10). SiRNA for APE-1 was treated to determine the role of APE-1 in the regulation of COX-2 expression, VEGF production and anti-apoptotic effect against cisplatin.

Results In human ESCC tissues, nuclear localization of APE-1 was observed in 92.3% (60/65) of all tissues. There was a significant relationship (p=0.029, R=0.49) between nuclear APE-1 and cytoplasmic COX-2 expression levels in the esophageal cancer tissues. In KYSE 220 and EC-GI-10 cells, MCP-1 stimulation significantly increased mRNA and protein expression of APE-1. Treatment with siRNA for APE-1 significantly inhibited p-STAT3 expression levels in MCP-1-stimulated both cells. Furthermore, treatment of siRNA for APE-1 significantly reduced COX-2 expression and VEGF production in MCP-1-stimulated esophageal cancer cell-lines. Treatment with APE-1 siRNA significantly increased apoptotic levels in cisplatin-incubated KYSE 220 and EC-GI-10 cells.

Conclusion APE-1 is overexpressed and associated with COX-2 expression and
VEGF production in esophageal cancer tissues.

**Key Words:** APE-1, Esophageal cancer, COX-2, VEGF, MCP-1
**Introduction**

Esophageal carcinogenesis induced by ethanol and smoking is closely related to the metabolism of ethanol, acetaldehyde and acetate, and the carcinogens in smoke, such as polycyclic aromatic hydrocarbons and N-nitrosamines, respectively. These factors generate cytokines, prostaglandins, and reactive oxygen/nitrogen species (ROS) in esophageal tissues. The host response to free radical-induced damage includes the induction of DNA repair enzymes, such as apurinic/apyrimidinic endonuclease -1 (APE-1) (7, 21). APE-1 is also a multifunctional protein such as a redox effector for several transcriptional factors including AP-1, HIF1-α and p53 (1). The presence of AP sites is known to block DNA synthesis or lead to mutations or genetic instability (20). If the function of BER is suppressed due to reduced APE-1 activity, sensitivity to chemoradiotherapy is increased (32). In addition, Zou et al have reported that APE-1-specific inhibitor, E3330 reduced tumorigenesis through inhibition of VEGF production (37). Then, there is no available data about the APE-1 expression and distribution in esophageal squamous cell carcinoma.

Cyclooxygenase-2 (COX-2) protein plays important roles in development of gastrointestinal cancers (34). Many studies have been reported that COX-2 is associated with tumorigenesis through angiogenesis and reduction of apoptosis (5, 29, 31). We have also reported that a selective COX-2 inhibitor significantly reduced the incidence of gastric cancer in MNU-treated *H. pylori*-infected Mongolian gerbils (8). In esophageal cancer tissues, COX-2 and VEGF as well as MCP-1 was one of predictive factors for severe prognosis and resistance against chemotherapy in esophageal cancer tissues (4, 24, 36). Moreover, a selective COX-2 inhibitor, celecoxib also inhibited APE-1 expression through reduction of IkB α
phosphorylation (8). Therefore, it is critical issue for understanding the precise
mechanism for development of esophageal cancer to clarify the relationship between
COX-2 and APE-1 expression. In addition, the MCP-1 receptor, CCR2 also has been
reported to impact on angiogenesis and VEGF production (23, 26) as well as MCP-1
which plays a pivotal role in tumor vascularity of esophageal cancer (24).

Given the role that APE-1 may play in the development of esophageal cancer, we
examined the co-expression of APE-1 and COX-2 expression as one predictive factor
in esophageal cancer tissues and determined whether APE-1 was associated with
VEGF production in the development of esophageal cancer tissues through induction
of COX-2 protein.
Materials and Methods

Patients

A total of 65 paraffin-embedded esophageal squamous cell carcinoma (ESCC) tissues were obtained from archived esophageal cancer patients who had undergone esophagectomies and/or received chemoradiotheraphy in Nippon Medical School Hospital. The protocol of this study was approved by the Ethics Committee of Nippon Medical School and written informed consent was obtained from all patients. Among these patients, there were 47 males and 18 females, ranging in age from 50-86 years. 65 tissues were categorized according to UICC (International Union Against Cancer) TNM classification as follows: 3 stage 0, 10 stage I, 10 stage IIA, 1 stage IIB, 19 stage III, 5 stage IVA, 17 stage IVB.

Cell culture and treatments

Esophageal squamous cell carcinoma (ESCC) cell lines, KYSE 220 and EC-GI-10 (purchased from National Institute of Biomedical Innovation, Japan) was maintained in RPMI 1640 or Ham F12 (Nikken Bio Medical Laboratory, Japan) supplemented with 10% fetal bovine serum (Nippon Bio-Supply Center, Japan) at 37°C in 5% CO₂. Group 1 without any treatments served as the control, group 2 was stimulated by hr MCP-1 (human recombinant monocyte chemoattractant protein-1, 0.1μmol/l) without pretreatment of siRNA for APE-1, while group 3 was stimulated by MCP-1 (0.1μmol/l) after 24hr pretreatment with siRNA for APE-1. Then, cisplatin (0, 1, 3, and 6μl/ml) was added to the KYSE220 cells with or without pretreatment of siRNA for APE-1.
Immunohistochemical analysis of APE-1, COX-2, MCP-1, CCR2 and VEGF

Expression of APE-1, COX-2, MCP-1, CCR2 and VEGF in the esophageal cancer tissues was evaluated by immunostaining. Briefly, 4-μm sections were deparaffinized, antigens were retrieved by microwaving for 5 min in 5% urea solution, and endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol. The samples were incubated overnight at 4°C with rabbit anti-APE-1 antibody (diluted 1:100; Santa Cruz; USA), mouse anti-COX-2 antibody (diluted 1:150; Cayman; USA), rabbit anti-MCP-1 antibody (diluted 1:100; Abcam; USA), anti-CCR2 antibody (diluted 1:20; Abcam; USA) and anti-VEGF antibody (diluted 1:200; Santa Cruz; USA). After washing, the secondary antibody was detected by LSAB 2 kit (DAKO) with diaminobenzidine as chromogen. For the negative control, primary antibodies were replaced with isotype-matched immunoglobulin.

Double labelling immunofluorescence methods and confocal laser scanning microscopy were used to evaluate the co-expression of immunoreactivity for the pair of mouse anti-human COX-2 (diluted 1:40; Cayman; USA) and rabbit anti-human APE-1 (diluted 1:20; Santa Cruz; USA). Sections were incubated overnight at 4°C with a mixture of the two primary antibodies, and then with FITC or Texas red conjugated secondary antibodies (horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) and goat anti-rabbit IgG (Vector), for COX-2 and APE-1, respectively) followed by nuclear counterstaining with 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO).

The immunostaining scoring system as follows was established by experienced pathologists. For APE-1, immunoreactivity was graded from 0 to 3 (0:0%, 1:0~33%,...
2:33~66%, 3:67~100%) based on the proportion of positive nuclei to total cell number in representative areas of the cancer tissue (9). For COX-2, immunoreactivity was determined by the intensity of staining and the percentage of areas with positive staining. The intensity of staining was graded from 0 to 3, and the area of positivity was estimated as percentage of total area of the tumor. These two variables constituted the actual score: 0, 1, 2, 3, as described previously (22). Six fields were selected to determine the average score of APE-1 and COX-2. For MCP-1, sections with more than 30% positive cells were considered as positive, while the sections with 30% or less positive cells were considered as negative, as described previously (24). Since Kitadai et al have reported that MCP-1 was significantly correlated with VEGF immunoreactivity in esophageal cancer tissues (positive staining for VEGF was defined as the presence of VEGF immunoreactivity>30% of esophageal cancer cells), we have also determined the definition of MCP-1 positivity based on previous studies (24, 17).

**Western blotting analysis of APE-1 and p-STAT3 expression levels in treated KYSE 220 and EC-GI-10 cells**

Stimulated (H₂O₂: 200 μmol/l; MCP-1(0.03, 0.1 and 0.3 μmol/l)) KYSE 220 and EC-GI-10 cells were lysed in a buffer solution containing 0.1% NP-40, 10nM NaCl, 5mM MgCl₂, 10nM Na₂HPO₄, 65mM Na-orthovanadate and protease inhibitor cocktail. APE-1 protein, partially purified, was visualized by western blotting. Briefly, equal amounts of protein (20 μg) was analyzed by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in TBST and incubated overnight at 4°C with rabbit anti-APE-1 (diluted 1: 200; Santa
Cruz; USA), mouse anti-p-STAT3 (diluted 1:100; Santa Cruz) and mouse anti-β-actin (diluted 1:1000; Sigma; USA) antibodies, and after washing they were incubated with peroxidase-conjugated secondary antibodies (diluted 1:1000; GE Healthcare; UK) for 1 hour at room temperature. Electrochemiluminescence (ECL; GE Healthcare; UK) was used for detection.

Real-time PCR of APE-1 expression levels in treated KYSE 220 and EC-GI-10 cells

Real-time quantitative PCR was performed to detect mRNA expression levels of APE-1 from MCP-1-stimulated KYSE 220 and EC-GI-10 cells. Briefly, RNA extracted from these cells was reverse-transcribed, and subsequently cDNA was amplified in 7500 Fast real-time PCR system (Applied Biosystems; USA) with primers, dual-labeled fluorogenic probes, and a Taqman PCR Reagent kit (Applied Biosystems; USA). The primers and probes for APE-1 (Accession number: Hs00172396-m1*), COX-2 (Accession number: Hs00153133-m1*) and β-actin (Accession number: Hs99999903-m1*) were purchased from Applied Biosystems in USA. Known concentrations of serially diluted APE-1, COX-2 and β-actin cDNA generated by PCR were used as standards for quantification of sample cDNA. Copy numbers of cDNA for APE-1 and COX-2 were standardized to that of β-actin for the same sample.

RNA interference siRNA-APE-1 in KYSE220 and EC-GI-10 cells

For siRNA transfection, KYSE 220 and EC-GI-10 cells were inoculated at the density of 6×10^4/ml in RPMI-1640 medium without 10% fetal bovine serum and antibiotics. A cell suspension of 1.0 ml per well was seeded in 24-well plates.
siRNAs were synthesized corresponding to human APE-1 (Accession numbers: HSS 100555; HSS 100556; HSS 100557; Invitrogen Corp., USA), and one non-silencing siRNA was supplied as a control (control siRNA). The non-silencing control siRNA was synthesized using scrambled sequences.

Transfection of siRNAs and control siRNA into KYSE 220 and EC-GI-10 cells was performed using Lipofectamine RNAiMAX (Invitrogen Corp., USA). Briefly, 6 pmol siRNAs (final concentration 10-50nM) and 1.5 μl of Lipofectamine were used for each well. siRNAs and Lipofectamine were first diluted in Opti-MEM I Reduced Serum Medium (Invitrogen Corp., USA) respectively, and then, mixed and incubated for 20 min at room temperature for complex formation. Next, the entire mixture was added to the cells in the wells. At 24h after transfection, the cells of 24-well plates were used for real-time PCR for COX-2.

**Assessment of apoptosis**

To determine apoptosis, stimulated-KYSE220 and EC-GI-10 cells were treated with terminal deoxynucleotidyl transferase (TdT) enzyme with incubation in a humidified chamber at 37°C for 1 hr. Apoptosis was evaluated using terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay analysis (ApopTag; Oncor, Gaithersburg, MD, USA). To evaluate the degree of apoptosis, the number of TUNEL-positive cells was counted within a X 400 field. Four fields were selected to determine the average score count. Data were expressed as the mean percentage of total cell number (apoptotic index).

**Statistical Analysis**
Mann-Whitney U-test was used for analysis of categorical data, and Student’s T-test was used for analysis of continuous data. One-way ANOVA was used for multiple comparisons. Logistic regression analysis was used to determine factors associated with high APE-1 expression. Results were expressed as the mean±SD, and a p-value less than .05 was considered a statistically significant difference.
Results

Localization of APE-1, MCP-1, CCR2, COX-2 and VEGF in the esophageal cancer tissues

In human ESCC tissues, nuclear localization APE-1 was observed in 92.3% (60/65) of all tissues (Fig. 1A). In contrast, in normal esophageal tissues, APE-1 expression could not be observed (Figure 1B). COX-2, MCP-1 and CCR-2 were primarily expressed in the cytoplasmic compartment in the esophageal cancer cells (Fig. 1C, 1D and 1E). VEGF was also expressed in the cytoplasm in esophageal cancer cells (Fig. 1F). Specialized columnar epithelial cells could be seen in Barrett’s epithelium (Fig. 1G). APE-1 was expressed at low levels in specialized columnar epithelial cells in Barrett’s epithelium (Fig. 1H). In addition, staining could not be seen in esophageal cancer tissues using control IgG antibody (Fig. 1I).

COX-2 and APE-1 protein expression levels in MCP-positive esophageal cancer tissues

Since MCP-1 has been reported to play important roles in tumorigenesis in esophageal cancer tissues, we determined whether MCP-1 was associated with APE-1 and COX-2 expression in esophageal cancer tissues. In immunohistochemical analysis, both APE-1 and COX-2 expression scores in MCP-1-positive esophageal cancer tissues (n=41) were significantly (p<0.01, p<0.01, respectively) greater when compared to MCP-1-negative esophageal cancer tissues (n=24) (Figure 2).

Correlation between COX-2 and APE-1 expression in esophageal cancer tissues
Since APE-1 and COX-2 were strongly expressed in the esophageal cancer tissues, in this study, we determined the relationship between COX-2 and APE-1 expression levels in esophageal cancer tissues. FITC labeled (green) cells in the esophageal cancer tissues in Figure 3A show COX-2 immunoreactivity (Figure 3A). Texas red conjugated cells in Figure 3B show APE-1 immunoreactivity for the same section (Figure 3B). There were a few double positive staining cells (yellow cells) for the same section (Figure 3C). However, we have addressed that nuclear APE-1 and cytoplasmic COX-2 expressions were mainly co-expressed in the esophageal cancer tissues (Figure 3C). In addition, we observed a significant relationship (p=0.029, R=0.49) between COX-2 and APE-1 expression levels in sixty-five esophageal cancer tissues (Figure 3E). Moreover, we have also determined that there was a significant relationship (p=0.021, R=0.41) between APE-1 and CD34-positive cells in esophageal cancer tissues (Figure 3F).

**APE-1 expression levels in MCP-1-stimulated esophageal cancer cell-lines**

To clarify whether MCP-1 stimulation induced APE-1 expression in esophageal cancer tissues, we examined APE-1 expression levels induced by H$_2$O$_2$ and MCP-1 stimulation in esophageal cancer cell-line, KYSE 220 and EC-GI-10 cells by western blotting analysis. H$_2$O$_2$ (200 μmol/l) and MCP-1 (0.03, 0.1 and 0.3 μmol/l) stimulations increased APE-1 protein expression in both cell lines (Figure 4). APE-1 expression was also observed in 200 μmol/l H$_2$O$_2$ stimulated KYSE 220 and EC-GI-10 cells. APE-1 protein levels were low in unstimulated KYSE 220 and EC-GI-10 cells (Figure 4).
To clarify whether APE-1 action had an effect on STAT3 activation in esophageal cancer cells, we examined p-STAT3 expression levels in MCP-1-stimulated KYSE220 and EC-GI-10 cells treated with siRNA for APE-1. Treatment with siRNA for APE-1 completely suppressed APE-1 mRNA levels in KYSE 220 and EC-GI-10 cells by real-time PCR measurement (Figure 5). Treatment with siRNA for APE-1 significantly inhibited p-STAT3 expression levels in MCP-1 stimulated KYSE 220 and EC-GI-10 cells (Figure 6).

To determine the role of APE-1 in the regulation of COX-2 expression, we measured COX-2 mRNA levels in KYSE 220 and EC-GI-10 cells treated with siRNA for APE-1. MCP-1 stimulation significantly increased COX-2 mRNA expression levels compared to those in unstimulated KYSE 220 and EC-GI-10 cells as well as control non-silencing siRNA- and siRNA-treated cells. 5nM siRNA for APE-1 significantly reduced the expression of COX-2 mRNA levels and VEGF production in MCP-1-treated KYSE 220 and EC-GI-10 cells compared to in MCP-1-treated KYSE220 and EC-GI-10 cells (p<0.001)(Fig. 7A and 7B). SiRNA treatment for APE-1 could not significantly reduce COX-2 levels in unstimulated KYSE220 and EC-GI-10 cells (Figure 7A). In contrast, 5nM siRNA treatment for APE-1 also significantly inhibited VEGF production in KYSE 220 and EC-GI-10 cells compared
to those in control non-silencing siRNA treated and unstimulated cells (Figure 7B).

*Apoptosis levels in cisplatin-incubated esophageal cancer cell-lines treated with siRNA for APE-1*

To clarify the mechanism by which APE-1 activity was associated with sensitivity to chemoradiotherapy, we have investigated levels of apoptosis in cisplatin-incubated KYSE220 and EC-GI-10 cells with or without siRNA for APE-1. siRNA for APE-1 treatment significantly increased apoptotic levels in cisplatin (3μl/ml and 6μl/ml)-incubated KYSE220 cells (Figure 8A) and EC-GI-10 cells (Figure 8B).
Discussion

In this study, we aimed to investigate the localization of APE-1 expression in esophageal cancer tissues and to determine whether APE-1 was associated with development of esophageal cancer through elevation of COX-2 expression and VEGF production. Our major findings are: (1) APE-1 was overexpressed and found primarily in esophageal cancer cells with nuclear localization; (2) A significant relationship between APE-1 and COX-2 expression scores in esophageal cancer tissues was demonstrated; (3) MCP-1 stimulation significantly increased APE-1 protein level in KYSE 220 and EC-GI-10 cells; and (4) Reduction of APE-1 by specific siRNA significantly reduced COX-2 mRNA expression levels and VEGF production in MCP-1-stimulated KYSE 220 cells.

To our knowledge, this is the first report of APE-1 overexpression and a significant relationship between COX-2 and APE-1 in esophageal cancer tissues. Previous studies have shown that overexpression of APE-1 was associated with resistance to chemoradiotherapy in HeLa cells (12), glioma cell (27), medulloblastoma and primitive neuroectodermal tumors (3), as well as non-small cell lung cancer (33). Overexpression of APE-1 is found in many kinds of malignancies of digestive tract-associated organs, including cytoplasmic expression of APE-1 in colorectal cancer (16), and hepatocellular carcinoma (6), and nuclear expression in pancreatic cancer (15). Our results demonstrate that APE-1 was not detected in normal esophageal tissues, but it was expressed in 73.3% of cells in esophageal cancer tissues with nuclear localization of APE-1 was observed in 92.3% (60/65) of these tissues. The nuclear localization of APE-1 may be associated with chemoradiotherapy resistance and a poor survival (18).
Previous studies have reported that MCP-1 is expressed in esophageal cancer tissues and plays a pivotal role in tumorigenesis of esophageal cancer (24). Since we found that APE-1 expression levels in MCP-1-positive esophageal cancer were significantly increased compared to those with MCP-1-negative esophageal cancer, we investigated whether APE-1 expression levels were affected by MCP-1 stimulation using western blotting analysis. We showed that APE-1 expression in KYSE220 and EC-GI-10 cells was increased by MCP-1 stimulation. Our results suggest that MCP-1 may play an important role in APE-1 induction in esophageal cancer tissues. Further studies are needed to clarify the relationship between MCP-1 and APE-1 expression in esophageal cancer tissues.

In immunohistochemical analysis, there was a significant relationship between COX-2 and APE-1 in esophageal cancer tissues. We have previously reported that a selective COX-2 inhibitor, celecoxib inhibited APE-1 expression via reduction of IkBα phosphorylation (8). We have recently reported that MG-132, an NF-kB inhibitor, abrogated the p65 and APE-1 expression levels in MCP-1-stimulated esophageal cancer cell-lines (28). In addition, APE-1 has been reported to stimulate the DNA binding activity of NF-kB. Therefore, reduction of APE-1 in stimulated esophageal cancer cell-lines could lead to inhibition of stimulated COX-2 expression through downregulation of NF-kB activation. However in this study, basal COX-2 expression levels in unstimulated esophageal cancer cell-lines such as KYSE 220 and EC-GI-10 cells were not affected by siRNA treatment for APE-1 through reduction of DNA binding activity of NF-kB. Constitutive basal COX-2 expression levels in unstimulated esophageal cancer cell-lines might be partly induced in NF-kB-independent pathway as previous report (13). Previous studies have reported
that APE-1 interacts with HIF-1α, STAT3 and activates hypoxia-induced expression of VEGF (10, 35). In this study, we have also reported that inhibition of APE-1 expression was associated with reduction of p-STAT3 expression levels in stimulated esophageal cancer cell-lines. Zou et al have also reported that the APE-1-specific inhibitor, E-3330 inhibited tumorigenesis via reduction of growth of endothelial cells and VEGF production (37) and in another report, inhibition of APE-1 was associated with reduction of angiogenesis in vitro (14). In addition, Hall et al have also reported that APE-1 is also cytoprotective against endothelial apoptosis induced by hypoxia and by tumor necrosis factor-α (TNF-α), through the transcriptional upregulation of NF-kB-dependent survival signals, as well as NF-kB-independent mechanism (11). Previous studies and our study suggest that inhibition of APE-1 were partly linked to the reduction of VEGF production through reduction of activation of STAT3. In addition, although in our data, there was not co-localization of APE-1 and CD34 in esophageal cancer tissues, we have also demonstrated that APE-1 expression levels were significantly associated with CD34 expression levels in esophageal cancer tissues. Collectively, these results suggest that APE-1 may be modulated to prevent the development of esophageal cancer through reduction of angiogenesis.

APE-1 overexpression has been reported to be associated with resistance to various anti-cancer drugs. Several studies have shown that targeted reduction of APE-1 protein by specific anti-sense oligo or siRNA sensitized to MMS, H2O2, bleomycin and gemcitabine (3, 19, 25, 30). Whether such enhanced sensitivity is solely due to the loss of APE-1’s DNA repair activity or also due to the loss of its transcriptional regulatory function or both is still unknown. Our data showed that in APE-siRNA-treated KYSE220 and EC-GI-10 cells, the anti-apoptotic function of
APE-1 against cisplatin was abolished. Hall et al have reported that deletion of the redox-sensitive domain of APE-1 reduced the anti-apoptotic function of APE-1 in both hypoxia and tumor necrosis factor α induced endothelial cell death (11). Further, Bhattacharyya et al reported that *H. pylori*-mediated acetylation of APE-1 suppressed Bax expression and prevented p53-mediated apoptosis (2). Although there were a relatively small number (n=23) of patients treated with chemoradiotherapy, we found a negative trend (p=0.09, R=0.512) between effective ratio against chemoradiotherapy and APE-1 expression levels in esophageal cancer tissues. Further studies will be needed to clarify whether APE-1 expression is associated with chemo-resistance in esophageal cancer tissues. Taken together with these prior reports, our results suggest that APE-1 affects development of esophageal cancer through upregulation of VEGF production and reduction of apoptosis.

In conclusion, our data demonstrate that a significant relationship exists between APE-1 and COX-2 expression in esophageal cancer tissues. Moreover, reduction of APE-1 by specific siRNA significantly reduced COX-2 mRNA expression levels and VEGF production in MCP-1-stimulated KYSE 220 and EC-GI-10 cells. We believe that a greater understanding of the role of COX-2 in the regulation of APE-1 expression could provide potential future therapeutic targets in esophageal cancer.
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Figure Legends

Figure 1. Localization of APE-1, COX-2, MCP-1, CCR2 and VEGF in esophageal cancer tissues. (A) APE-1-, (C) COX-2-, (D) MCP-1-, (E) CCR2-, and (F) VEGF-positive cells in esophageal cancer tissues were identified by immunohistochemistry. (B) APE-1-positive cells were not detected in normal esophageal mucosa. (Magnification X125 for panels A, E, F, X150 for B, C and D). (G) Specialized columnar epithelial cells could be seen in Barrett’s epithelium (H&E staining; original magnification, x80). (H) APE-1 was observed at low levels in specialized columnar epithelial cells in Barrett’s epithelium (original magnification, x80). Arrows show APE-1 immunoreactivity in a faint nuclear pattern. (I) Staining was not seen in esophageal cancer tissues incubated with control IgG antibody (original magnification, x200).

Figure 2. COX-2 and APE-1 protein expression levels in MCP-1-positive esophageal cancer tissues

Expression levels of APE-1 and COX-2 in MCP-1-positive esophageal cancer tissues (n=41) were significantly higher compared to MCP-1-negative tissues (n=24) (p<0.001; p<0.001, respectively). Data are expressed as mean±SE of each score in sixty-five esophageal cancer tissues.

Figure 3. The relationship between APE-1 and COX-2 expression in esophageal cancer tissues

A. COX-2-positive cells (green cells) in the esophageal cancer tissues (original magnification, x150). B. APE-1-positive cells (red cells) in the esophageal cancer tissues (original magnification, x150). C. Nuclear APE-1 and cytoplasmic
COX-2 expressions were mainly co-expressed in the same section. Double positive cells (yellow cells) reveals COX-2-and APE-1-positive cells in the same section (original magnification, x150). D. 4',6-diamidino-2-phenylindole staining (blue cells) (original magnification, x150). E. There was a significant relationship (p=0.029) between APE-1 and COX-2 expressions in the sixty-five esophageal cancer patients. F. There was a significant relationship (p=0.021, R=0.41) between APE-1 and CD34-positive cells in the esophageal cancer tissues.

**Figure 4. APE-1 expression levels in MCP-1-stimulated KYSE 220 cells.**

APE-1 expression levels in H$_2$O$_2$ (200 μmol/l) and MCP-1(0.03, 0.1 and 0.3 μmol/l)-stimulated KYSE 220 and EC-GI-10 cells were significantly increased compared to unstimulated group.

**Figure 5 Treatment with siRNA for APE-1 in KYSE 220 and EC-GI-10 cells**

Treatment with 5nM siRNA for APE-1 in KYSE 220 and EC-GI-10 cells completely suppressed APE-1 mRNA levels. *p<0.01 vs control non-silencing siRNA treated KYSE 220 cells or unstimulated KYSE 220 cells. **p<0.01 vs control nonsilencing siRNA treated EC-GI-10 cells or unstimulated EC-GI-10 cells.

**Figure 6 p-STAT3 expression levels in stimulated KYSE 220 and EC-GI-10 cells treated with siRNA for APE-1**

p-STAT3 expression levels in MCP-1-stimulated KYSE 220 cells and EC-GI-10 cells treated with 5nM siRNA for APE-1 were significantly reduced compared to MCP-1-stimulated KYSE 220 and EC-GI-10 cells.
**Figure 7. Expression of COX-2 mRNA levels and VEGF production in KYSE 220 and EC-GI-10 cells treated with siRNA**

A. 5nM siRNA for APE-1 significantly reduced COX-2 mRNA expression levels in MCP-1-stimulated KYSE220 and EC-GI-10 cells. B. 5nM siRNA for APE-1 significantly reduced VEGF production in MCP-1-treated KYSE220 and EC-GI-10 cells. 5nM siRNA treatment for APE-1 also significantly inhibited VEGF production in KYSE 220 and EC-GI-10 cells compared to those in control non-silencing siRNA treated and unstimulated cells. Data are expressed as mean±SE of four separate experiments. *p<0.001 vs. 5nM siRNA for APE-1 treated cells, 5nM siRNA for APE-1 treatment in MCP-1-stimulated cells, control siRNA treated cells or unstimulated cells. **p<0.05 vs. Control siRNA treated cells or unstimulated cells.

**Figure 8 Apoptosis levels in cisplatin-incubated esophageal cancer cell-lines treated with siRNA for APE-1.**

(A) SiRNA for APE-1 treatment significantly (p<0.05) increased apoptotic levels in cisplatin (3μl/ml and 6μl/ml)-incubated KYSE220 cells. (B) SiRNA for APE-1 treatment significantly (p<0.05) increased apoptotic levels in cisplatin (3μl/ml and 6μl/ml)-incubated EC-GI-10 cells. Data are expressed as mean±SE of four separate experiments.
KYSE220 cells
p-STAT3
β-actin

EC-GI-10 cells
p-STAT3
β-actin

Control siRNA  siRNA  MCP-1  MCP-1 +siRNA