COX-2 Overexpression Induced by Gene Transfer Reduces Sensitivity of TE13 Esophageal Carcinoma Cells to 5-Fluorouracil and Cisplatin

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Abstract. *Previous clinicopathological studies demonstrated that overexpression of cyclooxygenase-2 (COX-2) is associated with a poor treatment response of esophageal carcinoma. The aim of this study was to elucidate the role of COX-2 overexpression in the chemosensitivity of esophageal carcinoma cells. TE13 human esophageal squamous cell carcinoma cells were transfected with a COX-2 constitutive expression vector, and stable transfectants overexpressing COX-2 were established. COX-2 overexpression in COX-2 transfectants was confirmed with western blotting and prostaglandin-E_2 (PGE_2) assay. Chemosensitivity testing revealed that sensitivity of COX-2 transfectants to 5-fluorouracil and cisplatin was significantly lower than in control vector-only transfectants, and that sensitivity of COX-2 transfectants was restored by the transfection of COX-2-specific siRNA. In addition, expression of antiapoptotic B-cell lymphoma-extra large (BCL-xL) and myeloid cell leukaemia-1 (MCL-1) was increased in COX-2 transfectants. These results indicate that COX-2 overexpression may reduce the chemosensitivity of esophageal carcinoma cells through up-regulation of the expression of antiapoptotic BCL-2 family proteins.*

Esophageal squamous cell carcinoma (ESCC) is one of the most malignant tumor types, and although the 5-year survival rate is still poor, the outcome of patients with ESCC has improved in recent decades through multimodal treatment (1). 5-Fluorouracil (5-FU) and cisplatin are central agents in chemotherapy and chemoradiotherapy (CRT) regimens in esophageal cancer treatment; however, there is a subgroup of tumors resistant to these treatments (2, 3).

Cyclooxygenase (COX), whose isoforms are known as COX-1 and COX-2, is an enzyme converting arachidonic acid to prostaglandin. COX-1 is constitutively expressed in normal tissues, and has been shown to be expressed similarly in tumor and normal tissues, whereas COX-2 is induced by cytokines and growth factors, and has been shown to be overexpressed in tumor tissues relative to normal tissues (4-6). Previous studies have shown that COX-2 overexpression in tumor tissues is associated with tumor progression, treatment resistance, and a poor prognosis in a variety of cancer types, including ESCC (7-13). In ESCC, COX-2 overexpression in pre-treatment biopsy specimens and post-surgical resection specimens has been shown to be associated with CRT resistance and a poor prognosis in patients with ESCC who underwent preoperative or definitive CRT (11-13). However, these results are based on immunohistochemical studies of COX-2 in clinical tumor specimens, and a causal relationship between COX-2 expression and CRT resistance has not yet been elucidated in an *in vitro* study with mechanistic considerations. On the other hand, previous studies of our group have demonstrated that COX-2 expression was significantly up-regulated in radioresistant esophageal carcinoma cell lines relative to the parental cell lines when mRNA expression profiles were compared (14). However, it remains unclear whether COX-2 overexpression may result in radioresistance.

In the present study, we performed an *in vitro* study to clarify the causal relationship between COX-2 expression

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and treatment response in terms of chemosensitivity, using COX-2 overexpression by gene transfection into TE13 esophageal carcinoma cells.

Materials and Methods

Cell culture. The human ESCC cell line, TE13, was obtained from the Cell Resource Center for the Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). The cells were maintained in culture medium (CM) consisting of RPMI-1640 (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Cell Culture Bioscience, Nichirei Biosciences, Tokyo, Japan), and incubated at 37˚C in 5% CO_2.

Establishment of COX-2 transfectants. Twenty-four hours after plating TE13 cells (1×10^5 cells/well) on 24-well plates in CM, cells were transfected with a COX-2 constitutive expression vector (pBOSNeoCOX-2) and control vector (pBOSNeo) (15) using lipofectamine (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After 24 h, the transfected cells were trypsinized from the plate and placed in a 10-cm culture dish in CM containing a final concentration of 1.0 mg/ml G418 (Geneticin; Invitrogen). The G418-resistant clones were then selected and expanded. The clones, which were confirmed to overexpress COX-2 by the methods described below [western blotting and prostaglandin-E_2 (PGE_2) assay], were used for chemosensitivity testing. The cells transfected with pBOSNeo were used as controls.

Enzyme immunoassay for PGE_2. Cells were cultured overnight in a 12-well plate at a density of 1×10^3 cells/well, and incubated at 37˚C for 15 min with 1 μM arachidonic acid (Sigma, St. Louis, MO, USA) in RPMI-1640. PGE_2 concentrations in the medium were then determined using an Endogen PGE_2 Competitive ELISA (Endogen, Cambridge, MA, USA), according to the manufacturer's instructions.

Western blotting. Antibodies against COX-2 (Cayman Chemical, AnnArbor, MI, USA), B-cell lymphoma-2 (BCL-2) (Cell Signaling Technology, Danvers, MA, USA), B-cell lymphoma-extra large (BCL-xL) (Cell Signaling Technology), myeloid cell leukaemia-1 (MCL-1) (Cell Signaling Technology), BCL-2 associated X protein (BAX) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology) were used in western blotting. Equal protein loading was evaluated by GAPDH. Cultured cells were collected and lysed with a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 1 mM EDTA, and a complete protease inhibitor cocktail tablet (cOmplete, Mini Protease Inhibitor Cocktail Tablets; Roche Applied Science, Indianapolis, IN, USA) for 60 min on ice. The extracts were sonicated three times each for 5 s at 20 kHz. The sonicated cell extracts were centrifuged at 20,630 ×*g* for 30 min at 4˚C, and supernatants were collected. Protein concentrations were determined by Protein Assay Rapid Kit Wako (Wako Pure Chemical Industries, Ltd. Osaka, Japan). Equal amounts of protein (50 μg) were mixed with sodium dodecyl sulfate (SDS) sample buffer containing 2-mercaptoethanol, boiled for 5 min, and loaded on 7% SDS-Poly-Acrylamide Gel Electrophoresis (PAGE) gels. Protein was transferred to polyvinylidene difluoride membranes (GE Healthcare, Milwaukee, WI, USA) with a tank transfer system (Bio-Rad, Hercules, CA, USA). The membranes for the antibody reaction were blocked in 5% skimmed milk at room temperature for 1 h. Membranes were reacted with specific primary antibodies diluted as follows: COX-2 1:500, BCL-2 1:1,000, BCL-xL 1:1,000, MCL-1 1:1,000, BAX 1:500, and GAPDH 1:500, at room temperature for 1 h. The bound primary antibody was detected by a secondary horseradish peroxidase-linked appropriate species antibody preparation. Signal detection was performed using an enhanced chemiluminescence (ECL) system (ECL Plus Western Blotting Detection Reagents; GE Healthcare).

Cell proliferation assay. Cell proliferation ability was evaluated by the WST-8 colorimetric assay. COX-2 and control transfectants (1×10^3 cells/well) were seeded into 96-well plates in 100 μl CM. After a 96-h incubation, 10 μl of WST-8 reagent solution (Cell Count Reagent SF; Nacalai Tesque Inc.) was added and cells were incubated for 1.5 h. Cell viability was determined by a colorimetric comparison in which optical density values were read from a microplate reader at an absorption wavelength of 450 nm (A_{450}).

Chemosensitivity testing. 5FU and cisplatin were generously provided by Kyowa Kirin (Tokyo, Japan) and Nippon Kayaku (Tokyo, Japan), respectively. Cells were plated at 1×10^3 cells/ml in 96-well plates in a volume of 100 μl and incubated for 24 h. The media were discarded and cells were incubated in the presence of graded concentrations of 5-FU (0.1, 1, 10, and 100 μg/ml) or cisplatin (0.1, 1, 10, and 100 μg/ml) for 72 h. Cell sensitivity to 5-FU and cisplatin was then evaluated by the WST-8 assay, as described above. The percentage of surviving cells was estimated by dividing the A_{450} of treated cells by the A_{450} of control untreated cells.

In the COX-2 inhibition experiment using siRNAs, transfectants (1×10^3 cells/well) were seeded on a 96-well plate in 100 μl CM and siRNA was transiently transfected as described below. After 24 h, the media were discarded. Cells were treated with graded concentrations of 5-FU and cisplatin for 72 h. Cell viability was determined by the WST-8 assay.

COX-2-specific inhibition by siRNAs. siRNA (siD324) specific for *COX-2* (GenBank Accession No. NM_000963) was prepared for COX-2 inhibition. siRNA and random siRNA (siD325) for the control were obtained from Takara Bio Incorporated (Otsu, Japan). Sense and anti-sense strands of siRNAs were as follows: (sequence siD324), 5'-CAAACGCUGAUUACAGAUATT-3', sense, and 3'-TTGUUUGCGACUAAUGUCUAU-5', antisense; siRNA-random (sequence siD325), 5'-UCUUAAUCGCGUAUAAGGCTT-3', sense, and 3'-TTAGAAUUAGCGCAUAUUCCG-5', antisense. All of these sequences were determined through a BLAST search (http://blast.ncbi.nlm.nih.gov/), to avoid sharing sequence homology with any known human mRNA. Transient transfection into *COX-2* transfectants was performed using Trans IT-TKO (Invitrogen).

Statistical analysis. Data are reported as the mean±standard deviation. The Student's *t*-test was used for statistical comparison. Differences were considered significant if a *p*-value less than 0.05 was obtained.

Results

Establishment and characterization of COX-2 transfectants. Several clones were confirmed to overexpress COX-2 according to the methods described above, and one representative clone, designated as TE13-COX-2 was used in

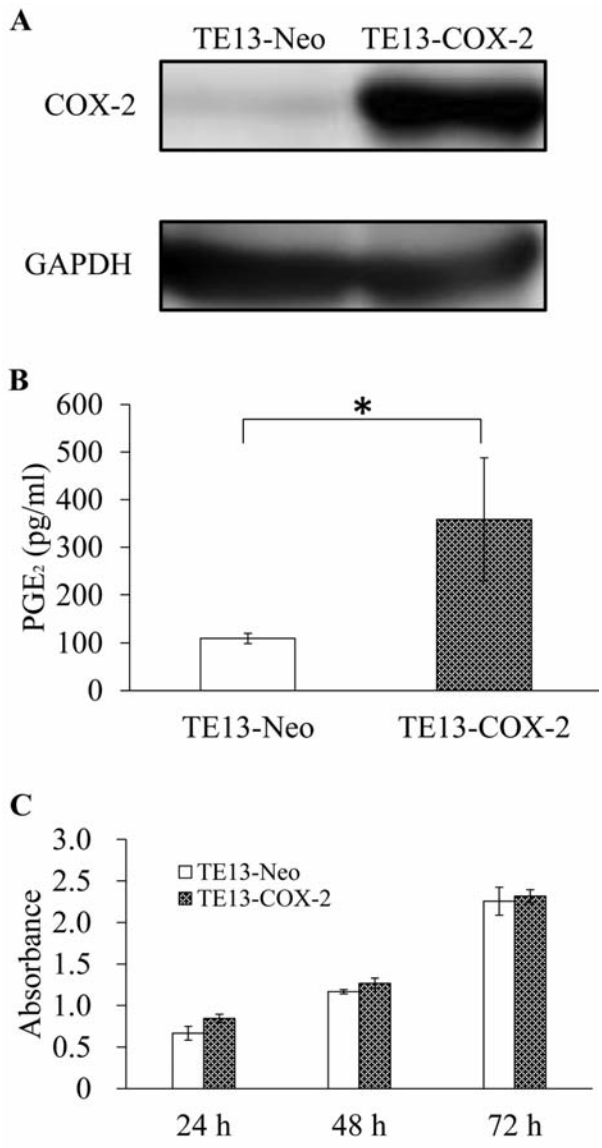

Figure 1. *Characteristics of cyclooxygenase-2 (COX-2) gene-transfected TE13 esophageal carcinoma cells. TE13 esophageal carcinoma cells were transfected with COX-2 cDNA and COX-2-overexpressing transfectants were established as described in Materials and Methods. Neomycin-resistant gene-transfected cells were used as control transfectants. A: Western blotting analysis of COX-2 expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. B: The prostaglandin E2 (PGE2) assay was used to evaluate for functional COX-2 expression. C: The WST-8 assay was used to evaluate the cell proliferation ability. Data represent the mean±standard deviation, n=6. *p<0.05. TE13-COX-2: COX-2-overexpessing transfectants, TE13-Neo: control transfectants.*

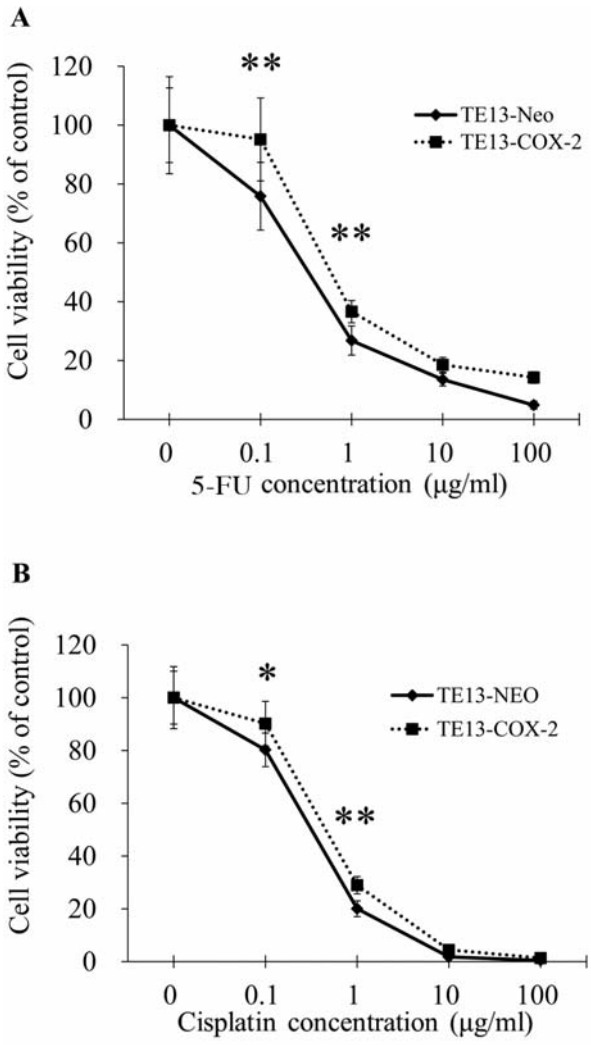

Figure 2. *Chemosensitivity of cyclooxygenase-2 (COX-2) gene-transfected TE13 esophageal carcinoma cells. A: Sensitivity of COX-2 gene-transfected esophageal carcinoma cells to 5-FU. B: Sensitivity of COX-2 gene-transfected esophageal carcinoma cells to cisplatin. Cell viability was determined by the WST-8 assay. Data represent the mean±standard deviation, n=6. *p<0.05, **p<0.01.*

subsequent experiments. In western blotting analysis, strong COX-2 expression was confirmed in TE13-COX-2, whereas slightly weak expression was detected in TE13-Neo (Figure 1A). COX-2 expression was also confirmed by the PGE_2 assay. Significantly higher levels of PGE_2 production were observed in TE13-COX-2 than in TE13-Neo cells (358.2±129.6 *vs*. 109.5±10.8 pg/ml, $p<0.05$) (Figure 1B). On the other hand, no significant difference in growth potential was observed between TE13-COX-2 and TE13-Neo cells by the WST-8 assay (Figure 1C).

Effect of COX-2 overexpression on chemosensitivity. Sensitivity to 5-FU was significantly lower in TE13-COX-2 cells than in TE13-Neo cells at concentrations of 0.1 μg/ml (95.2±4.1% *vs*. control: 75.8±3.3%, $p<0.01$) and 1.0 μg/ml (36.6±1.1% *vs*. control: 26.8±1.4%, $p<0.01$) (Figure 2A). In

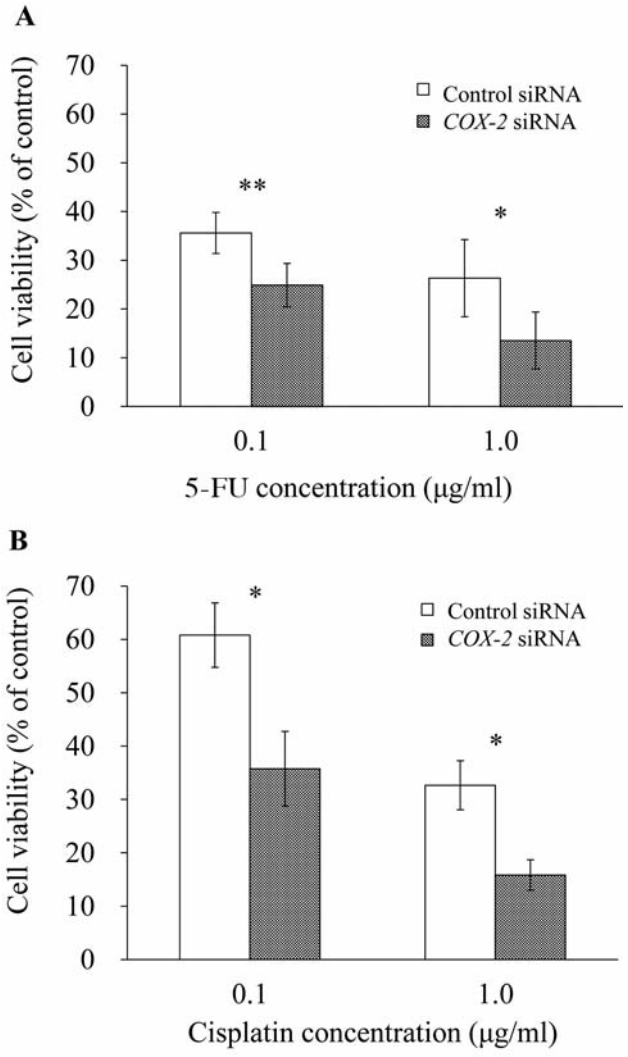

Figure 3. *Effect of cyclooxygenase-2 (COX-2)-specific siRNA on the chemosensitivity of COX-2 gene-transfected TE13 esophageal carcinoma cells. A: Effect of siRNA-mediated COX-2 suppression on the 5-FU sensitivity of COX-2 gene-transfected TE13 esophageal carcinoma cells. B: Effect of siRNA-mediated COX-2 suppression on the cisplatin sensitivity of COX-2 gene-transfected TE13 esophageal carcinoma cells. Cell viability was determined by the WST-8 assay. Data represent the mean±standard deviation, n=5, *p<0.05, **p<0.01.*

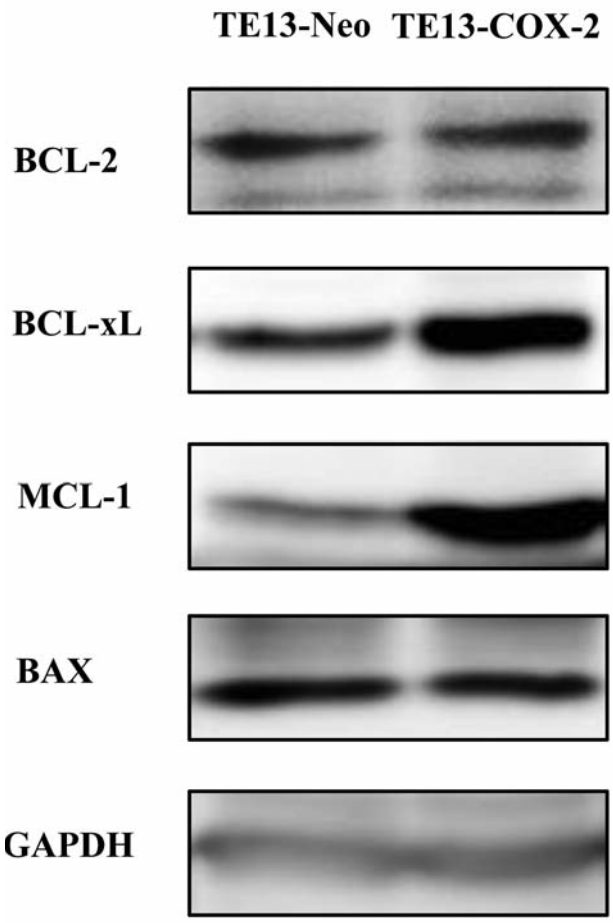

Figure 4. *Western blotting analysis of the B-cell lymphoma-2 (BCL-2) family protein expressions in COX-2 gene-transfected TE13 esophageal carcinoma cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. BCL-xL: B-cell lymphoma-extra large, MCL-1: myeloid cell leukaemia 1, BAX: BCL-2 associated X protein.*

addition, sensitivity to cisplatin was significantly lower in TE13-COX-2 cells than in TE13-Neo cells at concentrations of 0.1 μg/ml (90.2±3.2% *vs*. control: 80.2±2.2%, $p<0.05$) and 1.0 μg/ml (28.9±0.9% *vs*. control: 20.0±0.8%, $p<0.01$) (Figure 2B). Data represent the average absorbance of six wells in one experiment. The figure shown is representative of three independent experiments. Reduced sensitivity to 5-FU and cisplatin was observed in other clones confirmed to overexpress *COX-2* (data not shown).

Effects of COX-2 inhibition by siRNAs on chemosensitivity. Regarding sensitivity to 5-FU, at concentrations of 0.1 and 1.0 μg/ml of 5-FU, the viability of TE13-COX-2 cells, treated with control siRNA was 35.6±1.9 and 26.3±3.5%, respectively, whereas the viability of TE13-COX-2 cells treated with COX-2-specific siRNA was 24.9±2.5 and 13.5±2.6%, respectively. Cell viability was significantly reduced compared to control transfectants at each concentration ($p<0.05$) (Figure 3A). Regarding sensitivity to cisplatin, at concentrations of 0.1 and 1.0 μg/ml of cisplatin, the viability of TE13-COX-2 cells treated with control siRNA was 60.8±3.5 and 32.7±2.7%, respectively, whereas the viability of TE13-COX-2 cells treated with COX-2-specific siRNA was 35.8±4.9 and 15.8±2.0%, respectively, a difference which was significant at each concentration ($p<0.05$) (Figure 3B).

Expression of BCL-2 family proteins. To clarify the underlying mechanisms by which COX-2 overexpression reduces chemosensitivity of TE13 esophageal carcinoma cells, BCL-2 family protein expression (BCL-2, BCL-xL, MCL-1, and BAX) was examined by western blotting. Expression of antiapoptotic BCL-xL and MCL-1 was up-regulated in TE13-COX-2 cells relative to TE13-Neo cells, whereas expression of antiapoptotic BCL-2 and proapoptotic BAX was almost comparable in TE13-COX-2 and TE13-Neo cells.

Discussion

In the present study, COX-2 expression levels in TE13 esophageal carcinoma cells were genetically altered by transfecting *COX-2* cDNA or siRNA to appropriately evaluate the causal relationship between COX-2 expression levels and chemosensitivity. The results demonstrated that the overexpression of COX-2 contributes to the resistance to 5-FU and cisplatin and that COX-2-induced expression of antiapoptotic proteins plays a role in the resistance to these agents.

Selective inhibitors are conventional agents for COX-2 inhibition, and have been reported to suppress tumor growth or induce apoptosis *in vitro* and *in vivo* in esophageal carcinoma (16-20). Theoretically, these agents inhibit binding of COX-2 with arachidonic acid, thereby resulting in the inhibition of PGE_2 production, but do not inhibit COX-2 expression itself. However, the antitumor mechanisms of these agents are complex and not always COX-2-dependent; these agents have been shown to induce antitumor activities through PGE_2-independent mechanisms or affect COX-2 expression (19, 21). Therefore, we considered that the transfection approach is more suitable than use of conventional COX-2 inhibitors to precisely evaluate the relationship between COX-2 expression and treatment sensitivity.

COX-2 gene transfer into tumor cells has been reported to stimulate their growth *in vitro* or *in vivo*, and enhance the expression of angiogenesis or antiapoptosis factors (15, 22-23). In the present study, *COX-2* gene transfer into TE13 ESCC cells enhanced expression of antiapoptotic BCL-2 family proteins, but did not stimulate *in vitro* growth. The effect of COX-2 expression by gene transfer on tumor cells may vary, depending on differences in cell type or original COX-2 expression status.

Regarding the relationship between COX-2 expression and apoptosis resistance, COX-2 overexpression by gene transfer into tumor cells has been shown to up-regulate BCL-2, and MCL-1 expression (22, 23) and inhibit apoptosis induced by carboplatin, a platinum compound analogous to cisplatin (22). On the other hand, the BCL-2/BAX ratio has been reported to be correlated with reduced sensitivity to 5-FU (24). These findings strongly support the correlation between the up-regulation of antiapoptotic BCL-2 family proteins and reduced chemosensitivity of TE13 ESCC cells.

In conclusion, the present study demonstrated that COX-2 overexpression by gene transfer renders TE13 ESCC cells resistant to 5-FU and cisplatin, and that COX-2-induced expression of the BCL-2 family proteins plays a role in chemoresistance. The possible association of COX-2 overexpression by gene transfer with radioresistance remains to be resolved.

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