

***In vitro* susceptibility to anticancer agents of the human KB carcinoma cell line transfected with COX-2 cDNA**

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Abstract. In order to investigate the malignant phenotype of cyclooxygenase (COX)-2 overexpressing cancer cells, a human epidermoid KB carcinoma cell line minimally expressing COX-2 protein was transfected with human COX-2 cDNA. In this study, we used a COX-2 transfected clone KB/COX-2 and a neomycin-transfected clone KB/neo as the control. When we examined the susceptibility to anticancer agents, there was no difference between these two clones in vincristine, bleomycin and 5-fluorouracil, although KB/COX-2 showed a 2.5-fold resistance to cisplatin (CDDP) as compared with KB/neo. The IC₅₀ for CDDP was 4.3 μ M in KB/COX-2 and 1.7 μ M in KB/neo. Treatment with small interfering RNA (siRNA) mediated the inhibition of COX-2 significantly increasing the level of susceptibility to CDDP in COX-2 siRNA as compared to that of the control siRNA. The expression of MRP1 and MRP2 was stronger in KB/COX-2 than in KB/neo by Western blot analysis. In addition, apoptosis induction by CDDP was at a lower level in KB/COX-2 (31%) than in KB/neo (38%). These results suggested that the overexpression of COX-2 increases the intracellular production of MRP1 and MRP2 and causes drug resistance to CDDP.

Introduction

Cyclooxygenase (COX) catalyzes the synthesis of prostaglandins from arachidonic acid. Two isoforms of the COX enzyme exist, COX-1 and COX-2 (1-4). While COX-1 is expressed constitutively in many organs including the alimentary canal, COX-2 is induced by stimuli such as cytokines and growth factors and is involved in various biological responses. COX-2 is up-regulated at the sites of inflammation and in various cancer tissues; colon (2), stomach (3), breast (4), lung (5), esophagus (6), pancreas (7),

urinary bladder (8), prostate (9) and skin (10). We demonstrated that the overexpression of COX-2 elevated cell migration *in vitro* and tumorigenicity and local tumor invasion *in vivo* via up-regulating MMP and Rho family small GTPases and down-regulating TIMP activities in the human KB carcinoma cell line (11).

Cisplatin (CDDP) is one of the most potent anticancer agents, displaying clinical activity against a wide variety of human malignancies, including head and neck, testicular, lung, ovarian and colon cancers (12,13). Drug resistance is the major limitation on the use of this anticancer agent. The mechanisms responsible for CDDP resistance are multifactorial and thereby make the treatment difficult.

In this study, the human KB carcinoma cell line minimally expressing COX-2 protein was transfected with COX-2 cDNA and an isolated clone with high COX-2 expression was compared with a mock-infected clone in the susceptibility to anticancer agents of COX-2 transfected cells *in vitro*. In particular, the purpose of this study was to investigate the relationship between COX-2 expression and the level of susceptibility to CDDP.

Materials and methods

Cell line and cell culture. The human KB carcinoma cell line (14) derived from the epidermoid carcinoma of the floor of the mouth was used in this study. KB cells were grown in Dulbecco's modified Eagle's MEM (DMEM) (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA) and 4 mM L-glutamine as a growth medium at 37°C in a 5% CO₂ incubator. This cell line was routinely subcultured with an EDTA-trypsin mixture. KB cells, which minimally possessed COX-2 protein, were transfected with full-length human COX-2 cDNA (15) (a gift from Dr R. Kulmacz, University of Texas Medical School, Houston, TX) and pcDNA3 containing a neomycin-resistant marker by using a calcium phosphate method as described previously (16). We isolated one clone which expressed COX-2 the most as KB/COX and isolated the neomycin-transfected clone, KB/neo as a control (11).

Agents. Vincristine (VCR), bleomycin (BLM) and 5-fluorouracil (5-FU) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). CDDP was generously provided by

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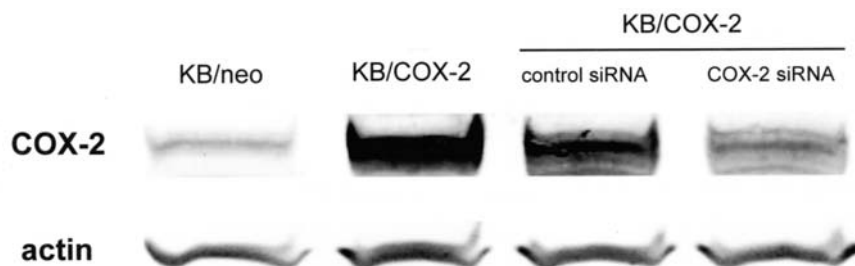


Figure 1. The expression of COX-2 protein in KB/COX-2, KB/neo, the control siRNA-treated KB/COX-2 and COX-2 siRNA-treated KB/COX-2 in Western blot.

Yakult Co. (Tokyo, Japan). VCR, BLM and CDDP were dissolved in distilled water and 5-FU in dimethylsulfoxide (DMSO) (Dojindo Laboratories, Kumamoto, Japan) before use.

Transfection of small interfering RNAs. KB/COX-2 cells were seeded with DMEM supplemented with 10% FBS for 24 h, then transfected with 5 nM of small interfering RNA (siRNA) using Lipofectamine™ 2000 (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's protocol. SMARTpool siRNA targeting COX-2 (siCOX-2; SMARTpool, M-004557-00) and control siRNA, Lamin A/C (siCONTROL Lamin A/C; SMARTpool D-001050-01-20) were purchased from Dharmacon Inc. (Lafayette, CO, USA). We used the cells at 24 h after transfection of siRNAs.

Cell growth assay. Cells were plated at 2.5×10^3 cells/well in a 100 μ l volume in 96-well plates and cultured in a growth medium at 37°C. Cell growth was assessed by 3(4,5-dimethylethiazolyl-2-)-2,5-diphenyl tetrazolium bromide (MTT) assay after 1, 3, 5 and 7 days of incubation, as described previously (17).

Cell growth inhibition by anticancer agents. Cells were plated at 2.5×10^3 cells/well in a 100 μ l volume in 96-well plates and cultured in a growth medium at 37°C for 24 h. The various concentrations of anticancer agents were added to the wells and then cells were cultured for a further 72 h. Cell growth was assessed by 3(4,5-dimethylethiazolyl-2-)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Western blot analysis. Cells were lysed in a lysis buffer composed of Mg^{2+} - and Ca^{2+} - free phosphate-buffered saline (PBS) containing 20 mM Tris-HCl, pH 8.0, 1% NP40, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% β -mercaptoethanol, 0.5 mM dithiothreitol and a mixture of proteinase inhibitors consisting of 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 5 μ g/ml leupeptin, 5 mM benzamidine, 1 μ g/ml pepstatin, 2 μ g/ml antipain hydrochloride (Boehringer, Mannheim, Germany), 50 μ M 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 2 mM sodium orthovanadate (Sigma-Aldrich) and 20 U/ml ulinastatin (Mochida Pharmaceutical, Tokyo, Japan). The lysate containing 15 μ g protein was electrophoresed in a 10-20% gradient SDS-PAGE mini gel (Bio-Rad, Chicago, IL, USA) and blotted onto a PVDF membrane using Multiphor II (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 30 min. The blotted

membrane was blocked with 5% skim milk in 10 mM Tris-HCl, pH 7.2, containing 150 mM NaCl and 0.5% Tween-20 and incubated with primary antibodies (0.1-1 μ g/ml) described below at 4°C for 16 h. The membrane was then incubated with alkaline phosphatase-conjugated secondary antibodies (0.02 μ g/ml) described below for 4 h at room temperature. The membrane was rinsed, treated with nitroblue tetrazolium (Sigma-Aldrich) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) to visualize protein bands. The primary antibodies used were goat polyclonal antibody against COX-2, MRP1 and CTR1, rabbit polyclonal antibody against ATP7A and ATP7B and mouse monoclonal antibody against MDR1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal antibodies against MRP2 (Alexis Co., Lausen, Switzerland). The secondary antibodies used were anti-goat, -rabbit or -mouse IgGs conjugated with alkaline phosphatase (Santa Cruz). Actin was used as an internal control.

Measurement of apoptosis. Cells were plated at 5×10^3 cells/well in a 96-well plate and grown at 37°C for 24 h. After a further treatment of 72 h of the cells with anticancer agents at the IC_{50} concentration, apoptosis was measured by a single-stranded DNA (ssDNA) apoptosis ELISA kit (Chemicon International, Inc., Temecula, CA, USA). Briefly, the cells were fixed in 80% methanol at room temperature for 30 min and dried up and formamide was added to each well. The plate was heated at 75°C for 10 min for DNA denaturation in apoptotic cells. After blocking with skim-milk, a mixture of primary monoclonal antibody to ssDNA and HRP-labeled anti-mouse IgM was added to each well and then incubated for 30 min at room temperature. The plate was then washed and incubated with peroxidase substrate ABTS [2,2'-AZINO-bis (3-ethylbenziazoline-6-sulfonic acid)]. Absorbance was measured in an ELISA plate reader at 405 nm and an apoptosis index (ratio of apoptotic cells to total cells) was determined.

Statistical analysis. Statistical analysis was performed by using the Student's t-test. Differences were considered significant at p -value <0.05.

Results

Isolation of cell clones overexpressing COX-2 protein. We isolated a clone where COX-2 expressed the most as KB/COX-2 and isolated the neomycin-transfected clone, KB/neo as the control. KB/COX-2 showed 3- to 4-fold increase of COX-2 protein expression as compared to KB/neo (Fig. 1). There was no apparent morphological change

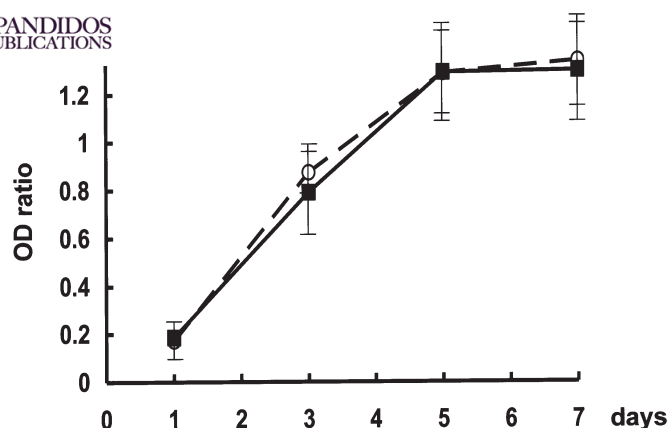


Figure 2. Growth curves of KB/COX-2 and KB/neo. Cells were plated at a density of 2.5×10^3 cells/well in a growth medium and incubated at 37°C in a 5% CO_2 incubator. Cell growth was determined by MTT assay at intervals. \circ ; KB/neo, \blacksquare ; KB/COX-2. Data represent mean \pm SD of 6 wells.

between KB/COX-2 and KB/neo. In the cell growth curve, KB/COX-2 and KB/neo showed a similar growth rate and there was no significant difference between them (Fig. 2).

Cell growth inhibition by anticancer agents. Anticancer agents induced growth inhibition in a dose-dependent manner in all the cell lines (Figs. 3 and 4). There is no difference between KB/COX-2 and KB/neo in VCR, BLM and 5-FU, though KB/COX-2 showed a resistance to CDDP as compared with KB/neo. The IC_{50} for CDDP was $4.3 \mu\text{M}$ in KB/COX-2 and $1.7 \mu\text{M}$ in KB/neo. Furthermore, we examined the effect of siRNA against COX-2 on susceptibility to CDDP. The level of susceptibility to CDDP was significantly increased in COX-2 siRNA as compared to the control siRNA (Fig. 4B).

Elevated expression of MRP1 and MRP2 in KB/COX-2. The transport system is an important mechanism of resistance to CDDP. Therefore, we examined the expression levels of proteins relative to the transport system. The expression of MRP1 and MRP2 was stronger in KB/COX-2 than in KB/neo by Western blot analysis, though there were no differences in the other proteins including MDR1, CTR1, ATP7A and ATP7B (Fig. 5). In particular, the expression of MRP2 was elevated by CDDP treatment.

Apoptosis induction by anticancer agents. Apoptosis was observed in KB/COX-2 and KB/neo after exposure of cells to anticancer agents. In this study, the apoptosis index for anticancer agents at the IC_{50} concentration was determined in the two cells. Although there was no significant difference in the apoptosis index for VCR, BLM and 5-FU between KB/COX-2 and KB/neo, the apoptosis induction of CDDP in KB/COX-2 was significantly weaker than that of CDDP in KB/neo ($p < 0.05$) (Fig. 6).

Discussion

The overexpression of COX-2 produces excess prostaglandins and causes an increase in cell proliferation and a decrease of apoptosis, to some extent mediated by the PGE2 receptor EP1-4

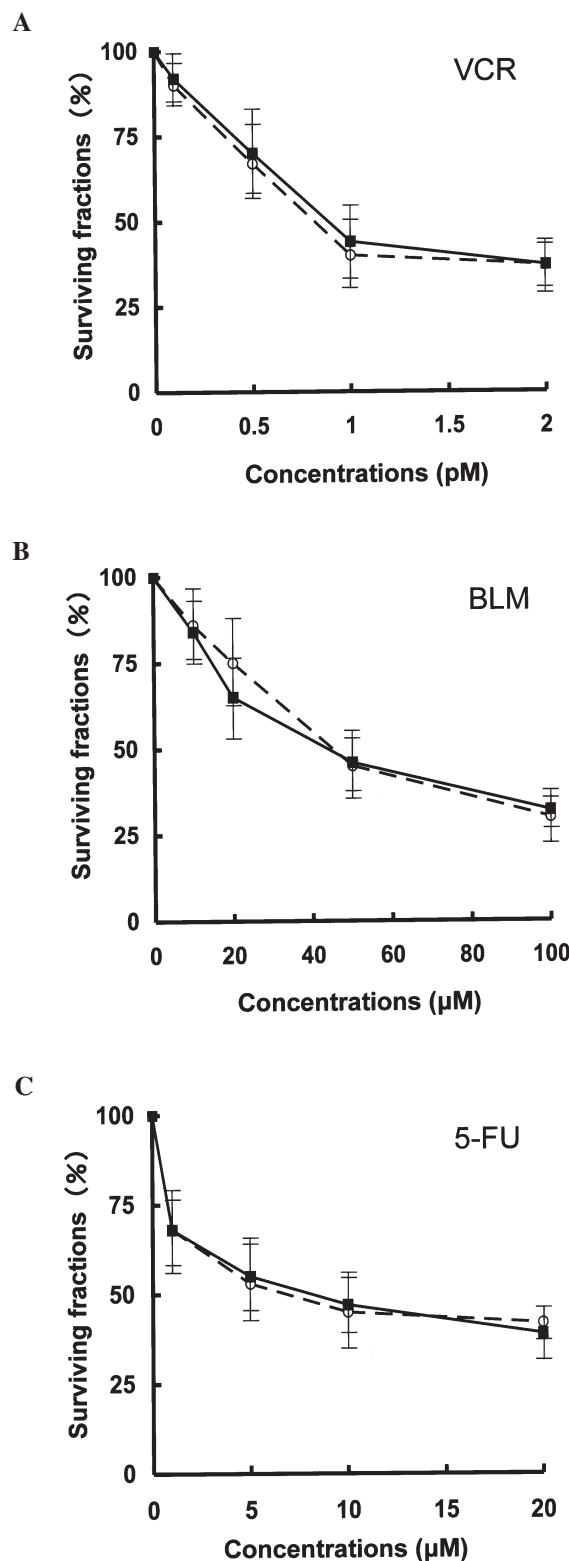


Figure 3. Cell growth inhibition by anticancer agents; (A) VCR, (B) BLM and (C) 5-FU in KB/COX-2 and KB/neo. Cell viability was determined using MTT assay after treatment of cells with various concentrations of anticancer agents at 37°C for 72 h. \circ ; KB/neo, \blacksquare ; KB/COX-2. Data represent the mean \pm SD of 6 wells.

(18). Many studies have indicated a significant involvement of COX-2 and PGE2 in carcinogenesis and progression for a variety of cancers (7,19,20). Torky *et al* showed that PGE2 induced MRP1 activity in cultured human lung cells (21).

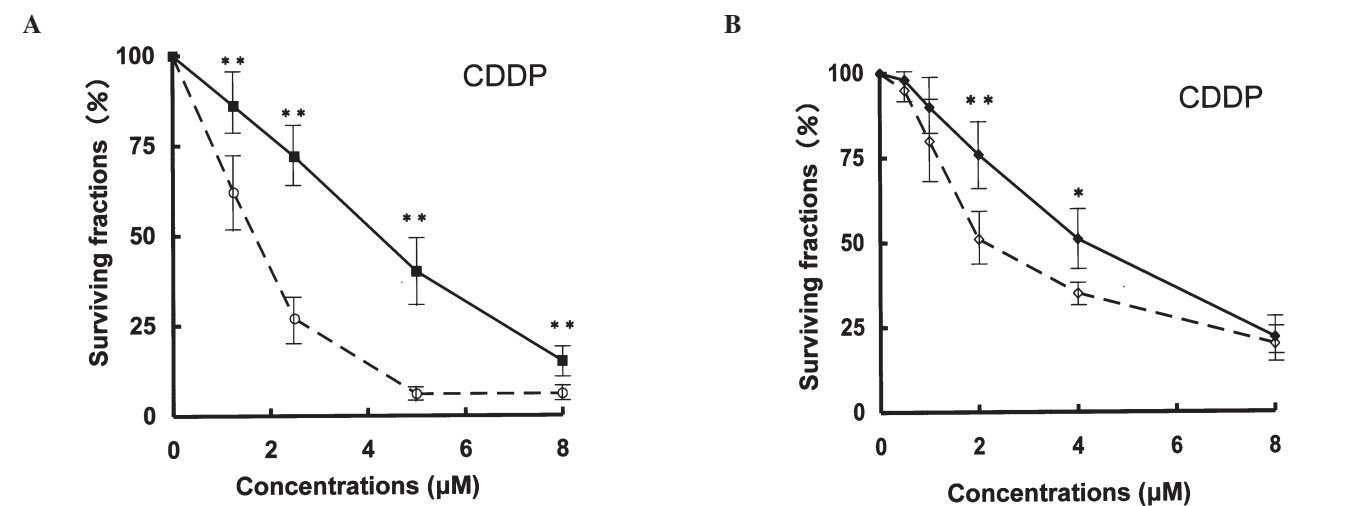


Figure 4. Cell growth inhibition by CDDP in (A) KB/COX-2, KB/neo, (B) control siRNA and COX-2 siRNA cells. Cell viability was determined using MTT assay after treatment of cells with various concentrations of anticancer agents at 37°C for 72 h. ○; KB/neo, ■; KB/COX-2, ◇; COX-2 siRNA, ◆; control siRNA. Data represent the mean ± SD of 6 wells. *P<0.05 and **p<0.005.

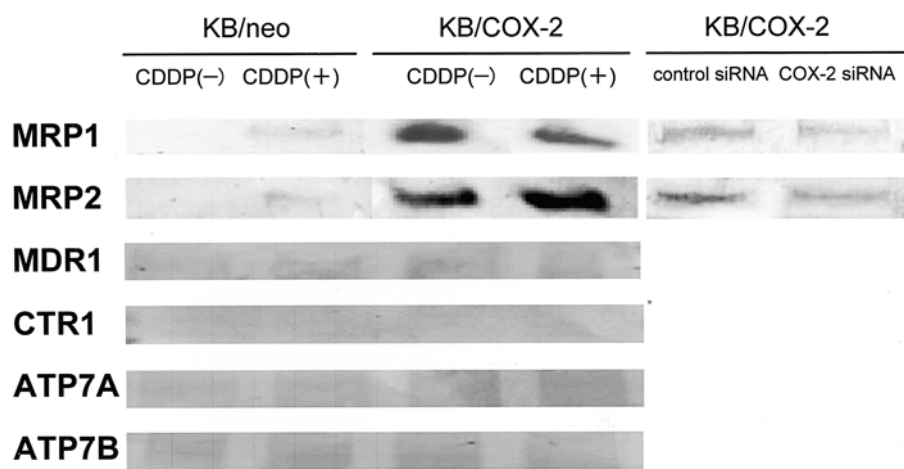


Figure 5. Expression levels of MRP1, MRP2, MRD1, CTR1, ATP7A and ATP7B were examined by Western blot using monoclonal or polyclonal antibodies against those proteins.

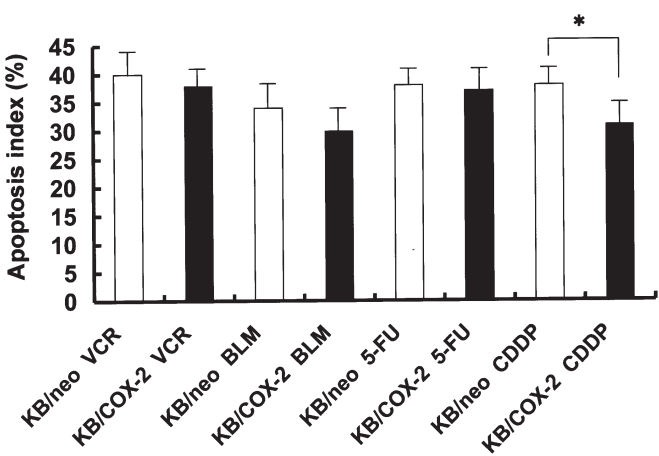


Figure 6. Apoptosis index by anticancer agents in KB/COX-2 and KB/neo. Both cell types were treated at 37°C for 24 h with anticancer agents at an IC₅₀ concentration for 72 h. The apoptosis index was determined using apoptosis EIA kits. *P<0.05.

COX enzymes and PGs might have an influence on the expression and function of MRP transporters during processes such as acute or chronic inflammation or various stages of cancer (21,22). Several studies have reported a relationship between COX-2 expression and molecular modifications in various molecules, including ABC transport proteins (MDR or MRPs) (23-26). In the present study, when we examined the susceptibility to anticancer agents, there was no difference between KB/COX-2 and KB/neo in VCR, BLM and 5-FU, though KB/COX-2 showed a 2.5-fold resistance to CDDP as compared with KB/neo. In a previous study, the over-expression of COX-2 increased intracellular production of MDR1 (23,24). On the contrary, Saikawa *et al* (27) reported that COX-2 overexpression induced an increase in MRP1 expression in a colon cancer cell line, though not MDR1. In our results, the expression of MRP1 and MRP2 was stronger in KB/COX-2 than in KB/neo by Western blot analysis, though there was no difference in MDR1. Many studies have been carried out in order to clarify the mechanisms and



regulating of CDDP resistance and different mechanisms have been elucidated. Reduced intracellular accumulation of CDDP depends on the balance of agent uptake and efflux is the most common and important factor of CDDP resistance (28). These processes are controlled by the number of transport proteins including the classic agent efflux pump P-gp (MDR1) and MRP1 (29). MRP2, like MRP1, belongs to the ABC transporter and functions as an ATP-dependent conjugate export pump (30). However, Taniguchi *et al* (31) reported that the overexpression of MRP1 does not confer resistance to CDDP. It is true that reduced intracellular accumulation of CDDP is the most common and important factor of CDDP resistance. Furthermore, there is evidence to suggest that a CDDP uptake might be mediated by the copper influx transporter CTR1 and the copper efflux transporters ATP7A and ATP7B (32,33). In this study, there was no difference in these copper-related transporters between KB/COX-2 and KB/neo.

The major goal of cancer chemotherapy is to commit tumor cells to apoptosis following exposure to anticancer agents. Their resistance is implied when tumor cells fail to undergo apoptosis. There is ample evidence indicating that reduced drug accumulation is a significant mechanism of CDDP resistance (33). In this study, the apoptosis induction of CDDP in KB/COX-2 was weaker than that of CDDP in KB/neo.

In summary, our results demonstrated that the overexpression of COX-2 increases the intracellular production of MRP1 and MRP2 and causes drug resistance to CDDP; hence it was strongly suggested that COX-2 regulating a malignant phenotype could become a potent therapeutic target for head and neck carcinoma.

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