Anti-proliferation and Apoptosis Induced by Selective COX-2 Inhibitor in Human Esophageal Squamous Carcinoma Cells

Hong-Ping Yu, PhD
Xiao-Li Wang, MD
Yan-Hua Su, MD
Yuan-Yuan Li, MD
Fang Li, MD
Shun-Qing Xu, PhD

Institute of Environmental Medicine, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, China

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ABSTRACT

The human esophageal squamous cell carcinoma (ESCC) cell line (EC9706), which expresses Cyclooxygenase-2 (COX-2), was used to investigate the mechanism by which a new nonsteroidal anti-inflammatory drug (NSAID), NS-398, inhibited proliferation and induced apoptosis in ESCC. The EC9706 cells were treated with various concentrations of selective COX-2 inhibitor, NS-398. Treatment with cox-2 selective inhibitor NS-398, significantly inhibited cell growth, induced apoptosis, and reduced the PGE_2 production in a dose-dependent manner in human esophageal squamous cell carcinoma cells. Further investigation showed that NS-398 could significantly increase caspase-3 activity, and the caspase specific inhibitor Ac-DEVD-CHO could inhibit the increase of caspase-3 activity induced by NS-398. Our results indicated that NS-398 could inhibit the proliferation, induce apoptosis in human ESCC cells by COX-2 dependant and COX-2 independent pathways. NS-398, a selective inhibitor of COX-2, may be a new method of chemoprevention and chemotherapy for ESCC.

INTRODUCTION

COXs are the key enzymes that mediate the production of prostaglandins (PGs) from arachidonic acid. Several reports indicated that levels of PGs in human tumors is higher than that of surrounding normal tissue and that the major PG is prostaglandin E_2 (PGE_2).[1,2] Two isoforms of COX have been identified, COX-1 and COX-2. COX-1 is expressed constitutively in most tissues, leading to relatively low levels of PG production; primarily, they perform a "housekeeping" function, such as gastric cytoprotection, maintenance of renal blood supply, and platelet aggregation. In contrast, COX-2 mRNA and protein are almost undetectable in most tissues, but can be induced by a number of agents including cytokines, growth fac-

tors, and tumor promoters. Increased COX-2 expression was observed in carcinomas of colon, stomach, breast, esophagus, lung, liver, pancreas, and bladder.[3-9] Increasing numbers of epidemiological and experimental studies have demonstrated that COX-2 play an important role in carcinogenesis of human cancers, and that non-steroidal anti-inflammatory drugs (NSAIDS) have an effect on the prevention of human cancers, especially those in the gastroenterological tract.[10,11] For example, individuals, who took NSAIDs regularly, had a 40% to 50% reduction in mortality from esophageal and colon cancers compared with those who did not use them.[12,13] Several NSAIDs, such as sulindac, celecoxib, were found to reduce the size and number of adenoma with familial adenomatous polyposis (FAP).[14-16] Furthermore, overexpression of COX-2 has been detected in human esophageal squamous dysplasia, esophageal squamous cell carcinomas and adenocarcinomas, and nonmalignant, metaplastic epithelium of Barrett's esophagus.[17-19] In addition, NSAIDs can reduce the frequency and number of pre-malignant and malignant lesions in animal models of esophageal carcinogenesis.[20] Experimental animal studies have shown that these drugs induce cancer cells to undergo apoptosis and inhibit carcinogenesis.[21,22] NSAIDs have also been shown to exert anti-proliferative and pro-apoptotic effects on a variety of cell lines, such as colon cancer cell lines, esophageal cancer cell lines, melanoma cell lines.[23,24] The mechanism by which COX inhibitors suppress carcinogenesis is partly attributed to its modulation of prostanoid production, which affects cell proliferation, tumor growth, and immune responsiveness. Recently, PGE_2, a major product of COX-2, has been reported to induce bcl-2 expression and inhibit apoptosis and, conversely, that COX-2 inhibitors induce apoptosis,[25] suggesting the possible mechanisms, COX-2- dependent and COX-2-independent in the action of anti-neoplasm. However, the molecular mechanisms by which NSAIDs prevent cancer in neoplastic cells, inhibit cell proliferation, and induce apoptosis remain unclear. NS-398, a selective COX-2 inhibitor, has been reported to inhibit proliferation and to induce apoptosis in colorectal and lung carcinoma cell lines.[26,27] In this study, the human ESCC cell line EC9706 was used as a model to explore the possible mechanisms of action of NS-398.

MATERIALS AND METHODS

Cell Culture

The human esophageal squamous cell carcinoma cell line EC9706 was supplied by Professor Ming-Rong Huang (Cancer Institute, Chinese Academy of Medical Sciences and Peking Union Medical University, Peking, China). EC9706 cells were plated in culture bottle and grown in PRMI-1640 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100μg/mL streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Reverse Transcription-PCR (RT-PCR)

Total cellular RNA was prepared from cell line EC9706 using a TRizol (Gibco) extraction technique. COX-2 transcript level was evaluated using a reverse transcription-PCR assay; □-actin transcript served as internal control. 1 μg of RNA samples was reversely transcribed in a 25 μL volume using Access RT-PCR Introduction System (Promega, San Luis Obispo, Calif), according to the manufacturer's instruction. 1 μL reverse transcription product was used for amplification with the following primers: 1) human cox-2 sense (5'-CAG CAC TTC ACG CAT CAG TT-3') and antisense (5'-TCT GGT CAA TGG AAG

CCT GT -3'), 756 bp; 2) β-actin sense (5'-CAC CCC CAC TGA AAA AGA TGA -3') and antisense (5'-CAT CTT CAA ACC TCC ATG ACG-3'), 326 bp. β-actin transcript served as internal control. PCR conditions consisted of 35 cycles of 94°C for 1 minute, 59°C for 1 minute and 72°C for 1.5 minutes, and a final extension at 72°C for 10 minutes. PCR products were visualized by 1.5% agarose gel electrophoresis.

Western Blot

EC9706 cells were lysed in solubilization buffer containing 150 mM NaCl, 50 mMTris (pH 8.0), 0.02% NaN_3, 0.01% PMSF, 0.2% aprotinin, 1% TritonX-100. Protein concentrations were determined, and 20 μg of protein per lane were electrophoresed on 10% SDS polyacrylamide gels after boiling for 5 minutes in 2× loading buffer, and transferred to nitrocellulose membranes. Native COX-2 protein was used as a positive control for COX-2 protein. The membrane was exposed to the anti-cox-2 antibody (1:500) overnight at 4°C, followed by incubation with the secondary antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, Calif). Blotted protein was stained with BCIP-DAB (SABC, Luoyan City, Henan, China).

Cell Proliferation Assay in Monolayer Cell Culture

The effect of NS-398 (Sigma) on cell growth was determined using a MTT cell proliferation assay. NS-398 was dissolved in 100% DMSO as 1000× stock solution and then diluted further in PRMI-1640 for cell culture experiments. The final concentration of DMSO for all experiments was maintained at 0.1%. All drugs solutions were prepared on the day of testing. EC9706 cells were seeded at a density of 5×10^3 per well in 96-well plates in PRMI-1640 containing 10% FBS. After 24 hours, the medium was then replaced with either a control medium containing 0.01% or a medium containing 25 μM NS-398, 50 μM NS-398, 100 μM NS-398, or 150 μM NS-398. After a 3-day preincubation, the cell proliferation assay, MTT, was performed. Absorbance was measured at 490 nm using a micro-ELISA reader.

DNA Fragmentation Analysis

DNA gel fragmentation assay was performed to detect EC9706 cell apoptosis. EC9706 cells were incubated in PRMI-1640 containing 10% FBS with 10 μM NS-398, 50 μM NS-398, or 100 μM NS-398, or without NS-398 for 2 days. DNA was extracted from 10^6 floating and attached cells. Briefly, cells were collected with cell scraper and washed with cold PBS. Cells were resuspended in TES solution (150 mM NaCl; 10 mM Tris, pH 7.5; 1 mM EDTA, PH 8.0; 1% SDS) for 5 minutes at room temperature. DNA was extracted with an equal volume of phenol, chloroform, isoamyl alcohol, and with phenol and isoamyl alcohol again, and then the aqueous phase was transferred to a fresh Eppendorf tube. Then, 1/10 volume of 3 M NaAc (PH5.2) and 2 volumes of 100% ethanol were added in the Eppendorf tube. After mixing well, DNA from the supernatant was precipitated by ethanol and resuspended in 100 μL of TE buffer (10 mM Tris, PH 8.0; 1 mM EDTA). The supernatant was treated with 50 μg/mL RNase A at 37°C for 1 hour. Equal amounts of DNA samples (20 μg) were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining.

Assay of Caspase-3 Activity

Cells were plated onto 24-well plates 1 day before treatment. After cells were exposed to 25 μM NS-398, 50 μM NS-398, 100 μM NS-398 for 2 days, both floating and attached cells were harvested and counted. Cells (1×10^6) were analyzed for caspase-3 activity using a

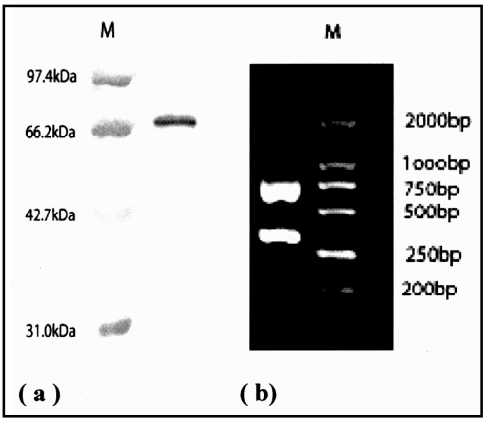

Figure 1. Expression of COX-2 in EC9706 cells cultured under normal conditions in vitro. (A) Western blot of cox-2 protein (70 kDa). (B) COX-2 mRNA expression in EC9706 cells (756 bp).

commercial kit (Roche). Experiments were performed in triplicate, and results were calculated as means ± SD.

Assay of PGE_2 Production

EC9706 cells were seeded at a density of 5×10^4 per well in 24-well plates in PRMI-1640 containing 10% FBS. After 24 hours, the medium was then replaced with medium containing 25 μM NS-398, 50 μM NS-398, 100 μM NS-398, or 150 μM NS-398. After a 24 hours preincubation, the supernatants were collected, and the PGE_2 level in the supernatants was measured using a commercial radioimmunoassay kit (Medical College, Suzhou University, China) according to instructions.

Statistical Analysis

All statistical analyses were performed by *t* test.

RESULTS

COX-2 Protein and mRNA Expression by EC9706 Cells

Figure 1A illustrates the expression of COX-2 protein (70 kDa), as determined by western blot analysis in human esophageal squamous cell carcinoma cell line (EC9706) under normal culture conditions. Figure 1B depicts the RT-PCR for detection of COX-2 mRNA (756 bp). A strong expression of COX-2 gene is apparent in EC9706 cells.

Effect of NS-398 on the Proliferation of EC9706 Cells

The effect of selective COX-2 inhibitor NS-398 on proliferation of EC9706 cells was studied by using the MTT cell proliferation assay. EC9706 cells were treated with increasing concentrations of NS-398 (25 μM, 50μM, 100 μM, 150 μM, and 200 μM), and the effect on proliferation was determined after 3 days of treatment. In cultures treated with NS-398, there was no increase in the number of non-adherent cells relative to control cultures. NS-398 suppressed the proliferation of the EC9706 cell examined in a dose-dependent manner (Figure 2).

Effect of NS-398 on Apoptosis of EC9706 Cells

To determine whether suppression of EC9706 cell proliferation by COX-2 inhibitors was due to the induction of apoptosis, the appearance of DNA fragmentation after incubation with NS-398 was detected by DNA gel fragmentation assay. As shown in Figure 3, NS-398-treated EC9706 cells produced a smear of different sizes of DNA fragments and a distinct oligosomal ladder, a characteristic of cells undergoing apoptosis. In contrast, control EC9706 cells showed no evidence of detectable DNA fragments.

Effect of NS-398 on PGE_2 Production by EC9706 Cells

Because PGE_2 is a major COX-2 product, the levels of PGE_2 produced by EC9706 cells were measured. PGE_2, produced by cells treated with NS-398, was significantly less than that without NS-398 treatment. As shown in Figure 4, NS-398 suppressed PGE_2 production in a concentration-dependent manner.

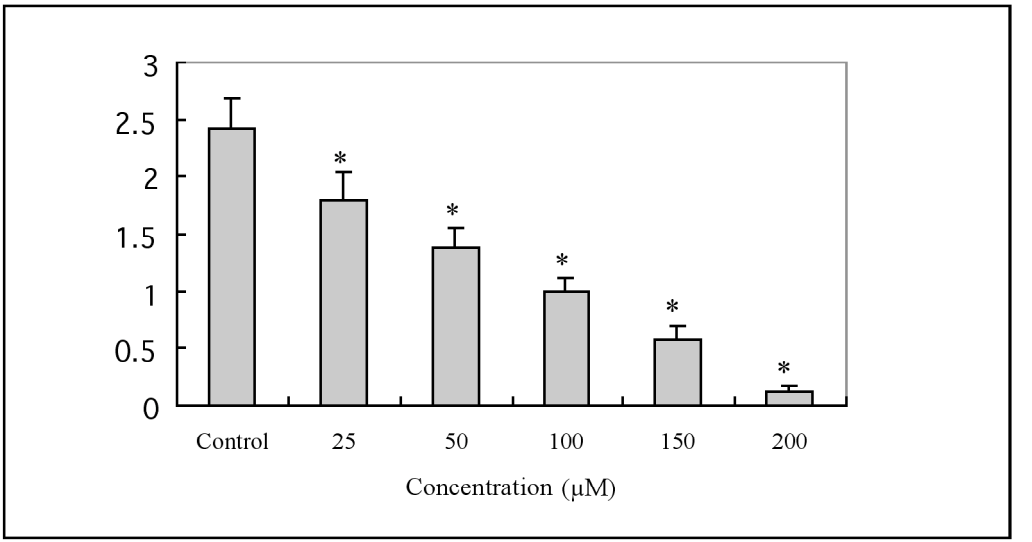

Figure 2. NS-398 inhibited EC9706 cell proliferation. EC9706 cells were treated with increasing concentrations NS-398 for 3 days. Data shown are means from 5 replicate wells.
*Significant difference at $P < 0.01$ vs control.

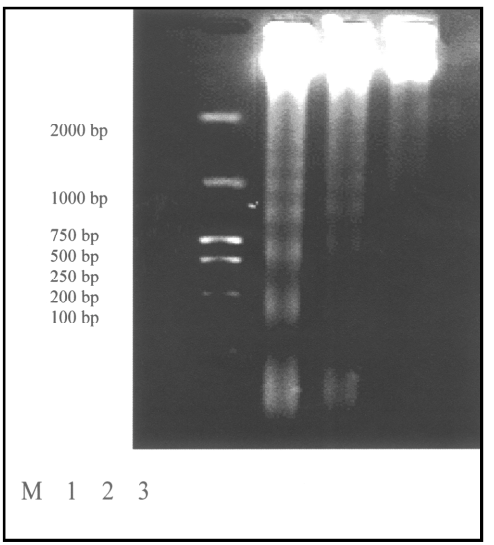

Figure 3. DNA-fragmentation assay. EC97-06 cells were treated with vehicle as control (Lane 3), 100 μM NS398 (Lane 2), 150 μM NS398 (Lane 1) for 2 days. Cellular DNA was extracted and analyzed by agarose gel (1.5%) electrophoresis. Lane M is DL-2000 marker.

Effect of NS-398 on Caspase-3 Activation in EC9706 Cells

Caspases are responsible for many of the biochemical and morphological changes that occur during apoptosis, so we investigated whether selective COX-2 inhibitor NS-398 induced the activation of caspase-3 in EC9706 cells. Caspase-3 activity was significantly elevated after 24 hours of treatment with different concentrations of NS-398. Furthermore, Caspase-3 activation was completely blocked by incubation with the caspase inhibitor Ac-DEVD-CHO (Figure 5).

DISCUSSION

To date, most studies concerning NSAIDs as chemopreventive agents for carcinogenesis have utilized NSAIDs, such as aspirin and sulindac, which inhibit both COX-1 and COX-2. Currently, there is also considerable interest in NSAIDs, which selectively inhibit COX-2, as they appear to be relatively non-toxic. Although only a few studies have been done, these newly developed NSAIDs appear, like celecoxib and NS-398, to be effective chemopreventives in animal models of neoplasia.[28,29] In fact, when evaluated using similar protocols, they are more effective than the following NSAIDs,

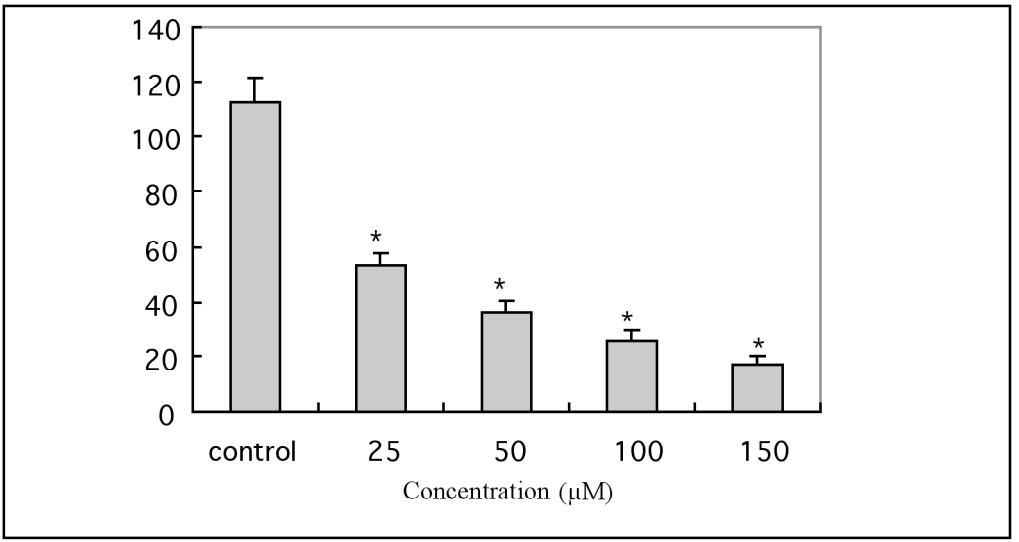

Figure 4. Effect of NS-398 on PGE_2 production by EC9706 cells. The cells were cultured without NS-398 (control), or else were incubated with 25 μM, 50 μM, 100 μM, 150 μM NS-398. The data shown are means ± SD of 5 determinations.
*Significant difference at $P < 0.01$ vs the control group.

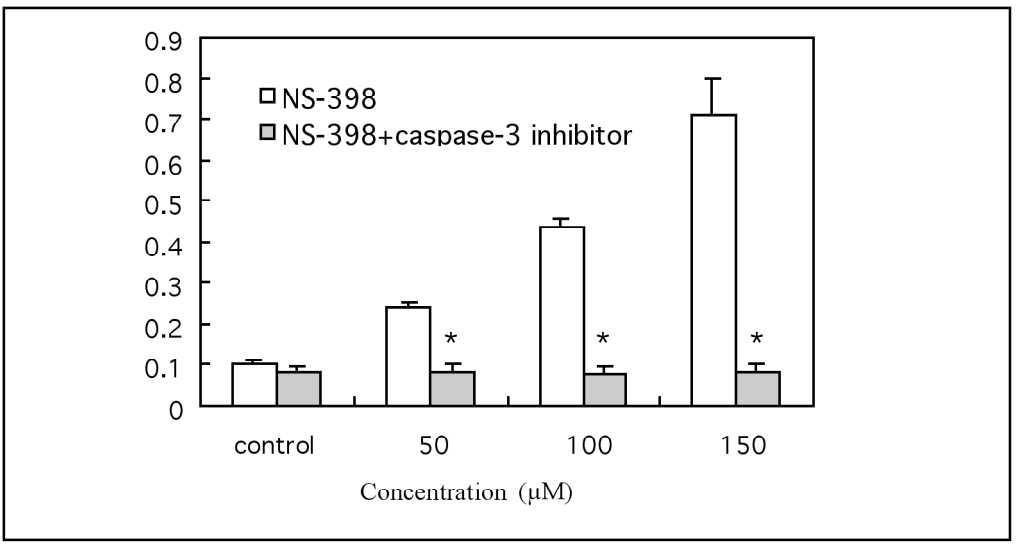

Figure 5. Effect of NS-398 on caspase-3 activation in EC9706 cells. The cells were cultured without NS-398 (control), or else were incubated with 50 μM, 100 μM, 150 μM NS-398. The data shown are means ± SD of 5 determinations.
*Significant difference at $P < 0.01$ vs the NS-398 group.

aspirin, sulindac, and ibuprofen. Substantial progress has been made in understanding the means by which NSAIDs, that are either non-selective for either COX isoform or selective for COX-1, are anti-neoplastic. Considerably less is known for highly selective COX-2 inhibitors.

Here, the effect of the highly selective COX-2 inhibitor NS-398 on human esophageal squamous cell carcinoma cell line EC9706 was tested. We have shown

that EC9706 cells express COX-2. NS-398 could strongly inhibit cell proliferation, and induce the characteristic features of apoptosis, including DNA fragmentation and caspase-3 activity. In our study, we found that NS-398 treatment of EC9706 cells for 24 hours resulted in elevation of caspase-3 activity in a dose-dependent manner, while caspase-3 activation was completely blocked by the caspase inhibitor, Ac-DEVD-CHO. Ming Li et al[23] also reported that induction of apoptosis by NS-398 is associated with caspase-3 activation, and the effect of NS-398 was inhibited by another caspase inhibitor Z-DEVD-FMK. These results imply that NS-398 could activate caspase-3 activity, which leads to apoptosis.

In addition, NS-398 could inhibit PGE_2 production that in turn may inhibit proliferation, and induce apoptosis in ESCC cells. A similar COX-2-dependent effect has been reported in colon, prostate, glioma, and pancreatic carcinoma cell lines,[8,22,28-31] where a selective COX-2 inhibitor was shown to inhibit proliferation and to induce apoptosis. Increased levels of prostaglandins (PGs), a consequence of COX-2 overexpression, play a role in tumor development by increasing cell proliferation, inhibiting cell apoptosis, promoting angiogenesis, and inhibiting immune surveillance.[32-34] Several other studies, however, have shown that there is no correlation between COX -2 expression and apoptosis induced by NSAIDs, including NS-398.[10,24] Elder et al[10] reported no correlation between the sensitivity of colon-cancer cell lines to NS-398 and COX-2 expression or the addition PGE_2 and the induction of apoptosis. This discrepancy may represent differences between the cells analyzed by different investigators and types of the NSAIDs used, and suggest more studies on the role of selective COX-2 inhibitor in cancer prevention and treatment should be progressed.

Because of non-selective COX -2 inhibitor's high frequency of side effects, long-term use is limited in the clinic.[35] Therefore, new and potent NSAIDs with fewer side effects, such as NS-398, celecoxib, rofecoxib, have been tested, and some of them have been used in the clinic. Our results found that NS-398 could inhibit human ESCC cells proliferation and induce esophageal cancer cells to undergo apoptosis, which supports the use of COX -2 inhibitors in esophageal cancer prevention and therapy. Our data provides a rational for the use of currently available selective inhibitors of COX –2, and these drugs may be effective preventive and therapeutic options for esophageal carcinoma.

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REFERENCES

1. Benerrt A, Civier A, Hensby CN, Melhuish PB, Stamford IF. Measurement of arachidonate and its metabolites extracted from human normal and malignant gastrointestinal tissues. *Gut*. 1987;28:315-318.

2. Katja CZ, Mario S, Artur-Aron W, Franz B, Helmut EG, Karsten S. Cyclooxygenase-2 expression in human esophageal carcinoma. *Cancer Res.* 1999;59:198-204.

3. Fujita T, Matsui M, Takaku K, Uetake H W, Taketo M, Sugihara K. Size- and invasion-dependent in crease in cyclooxygenase 2 levels in human colorectal carcinomas. *Cancer Res*. 1998;58:4823-4826.

4. Hwang D, Scollard D, Byrne J, Levine E. Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer. *J Natl Cancer Inst.* 1998,90:455-460.

5. Ristimaki A, Honkanen N, Jankala H, Sipponen P, Harkonen M. Expression of cyclooxygenase-2 in human gastric carcinoma. *Cancer Res.* 1997;57:1276-1280.

6. Hida T, Yatabe Y, Achiwa H, et al. Increased expression of cyclooxygenase-2 occurs frequently in human lung cancers, specifically in adenocarcinomas. *Cancer Res*. 1998;58:3761-3764.

7. Koga H, Sakisaka S, Ohishi M, et al. Expression of cyclooxygenase in human hepatocellular carcinoma: relevance to tumor dedifferentiation. *Hepatology.* 1999;29:688-696.

8. Tucker ON, Dannenberg AJ, Yang EK, et al. Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. *Cancer Res.* 1999;59:987-990.

9. Sweeney CJ, Marshall MS, Barnard DS, Heilman DK, Billing SD, Cheng L. Cyclooxygenase-2 expression in primary cancers of the lung and bladder compared to normal adjacent tissue. *Cancer Detect Prev.* 2002;26:238-224.

10. Elder Dj, Halton DE, Crew TE, Paraskeva C. Apoptosis induction and cyclooxygenase-2 regulation in human colorectal adenoma and carcinoma cell lines by the cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs NS-398. *Int J Cancer.* 2000;86:553-560.

11. Farrow DC, Vaughan TL, Hansten PD, et al. Use of aspirin and other nonsteroidal anti-inflammatory drugs and risk of esophageal and gastric cancer. *Cancer Epidemiol Biomarkers Prev.* 1998;7:97-102.

12. Taketo MM. Cyclooxygenase-2 inhibitors in tumorigensis (part II). *J Natl Cancer Inst.* 1998;90:1609-1620.

13. Funkhouser EM, Sharp GB. Aspirin and reduced risk of esophageal carcinoma. *Cancer.* 1995;76:1116-1119.

14. Smalley WE, Dubois RN. Colorectal cancer and nonsteroidal anti-inflammatory drugs. *Adv Pharmacol.* 1997;39:1-20.

15. Phillps RK, Wallace MH, Lynch PM, et al. A randomised, double blind, placebo controlled study of celecoxib, a selective cyclooxygenase 2 inhibitor, on duodenal polyposis in familial adenomatous polyposis. *Gut.* 2002;50:857-860.

16. Steinbach G, Lynch PM, Philips RK, et al. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med.* 2000;342:1946-1952.

17. Ratnasinghe D, Tangrea J, Roth MJ, et al. Expression of cyclooxygenase-2 in human squamous cell carcinoma of the esophagus; an immunohistochemical survey. *Anticancer Res.* 1999;19(1A):171-174.

18. Shamma A, Yamamoto H, Doki Y, et al. Up-regulation of cyclooxygenase-2 in squamous carcinogenesis of the esophagus. *Clin Cancer Res.* 2000;6:1229-1238.

19. Morris CD, Armstrong GR, Bigley G, Green H, Attwood SE. Cyclooxygenase-2 expression in the Barrett's metaplasia-dysplasia-adenocarcinoma sequence. *Am J Gastroenterol.* 2001;96:990-996.

20. Rubio CA. Antitumoral activity of indomethacin on experimental esophageal tumors. *J Natl Cancer Inst.* 1984;72:705-707.

21. Rubio CA. Further studies on the therapeutic effect of indomethacin on esophageal tumors. *Cancer.* 1986;58:705-707.

22. Tsujii M, Kawano S, Tsjui S, Sawaoka H, Hori M, Dubois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell.* 1998;93:705-716.

23. Li M, Wu XY, Xu XC. Induction of apoptosis by cyclooxygenase-2 inhibitor NS 398 through a cytochrome c-dependent pathway in esophageal cancer cells. *Int J Cancer.* 2001;93:218-223.

24. Molina,MA, Sitja-Amau M, Lemoine MG., Erazier ML, Sinicrope FA. Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: growth inhibition by nonsteroidal anti-inflammatory drugs. *Cancer Res.* 1999;59:4356-4362.

25. Sheng H, Shao J, Morrow JD, Beauchamp RD, Dubois RN. Modulation of apoptosis and Bcl-2 expression by prostaglandin E_2 in human colon cancer cells. *Cancer Res.* 1998;58:362-366.

26. Elder DJ, Halton DE, Hague A, Paraskeva C. Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2) selective nonsteroidal anti-inflammatory drug: independence from COX-2 protein expression. *Clin Cancer Res.* 1997;3:1679-1683.

27. Liu XH, Yao S, Kirschenbaum A, Levine AC. NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates bcl-2 expression in LNCcaP cells. *Cancer Res.* 1998;58:4245-4249.

28. Abou-Issa HM, Alshafie GA, Seibert K, Koki AT, Masferrer JL, Harris R. Dose-response effects of the COX-2 inhibitor, celecoxib, on the chemoprevention of mammary carcinogenesis. *Anticancer Res.* 2001;21:3425-3432.

29. Liu XH, Kirschenbaum A, Shen Y, Lee R, Holland JF, Levine AC. Inhibition of cyclooxygenase-2 suppresses angiogenesis and growth of prostate cancer in vivo. *J Urol.* 2000;164:820-825.

30. Elder DJE, Halton DE, Crew TE, Paraskeva C. Apoptosis induction and cyclooxygenase-2 regulation in human colorectal adenoma and carcinoma cell lines by the cyclooxygenase-2 selective non-steroidal anti-inflammatory drug NS-398. *Int J Cancer.* 2000;86:553-560.

31. Joki T, Heese O, Nikas DC, et al. Expression of cyclooxygenase 2(COX-2) in human glioma and in vitro inhibition by a specific COX-2 inhibitor, NS-398. *Cancer Res*. 2000;60:4926-4931.

32. Maxwell WJ, Kelleher D, Keating JJ, et al. Enhanced secretion of prostaglandin E_2 by tissue-fixed macrophages in colonic carcinoma. *Digestion.* 1990;47:160-166.

33. Zimmermann KC, Sarbia M, Weber AA, Borchard F, Gabbert HE, Schror K. Cyclooxygenase-2 expression in human esophageal carcinoma. *Cancer Res.* 1999;59:198-204.

34. Kokoglu E, Tuter Y, Sandikci KS, et al. Prostaglandin E_2 levels in human brain tumor tissues and arachidonic acid levels in the plasma membrane of human brain tumors. *Cancer Lett.* 1998;132:17-21.

35. Li M, Lotan R, Levin B, Tahara E, Lippman Sm, Xu X-c. Aspirin induction of apoptosis in esophageal cancer: a potential for chemoprevention. *Cancer Epidemiol Biomarkers Prev*. 2000;9:545-549.