PROGNOSTIC SIGNIFICANCE OF CYCLOOXYGENASE-2 IN BREAST AND ESOPHAGEAL CARCINOMA

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Academic Dissertation

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I Ristimäki A*, **Sivula A***, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H, Isola J: Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. Cancer Res, 2002;62:632-635.

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III Buskens CJ*, van Rees BP*, **Sivula A**, Reitsma JB, Haglund C, Bosma PJ, Offerhaus GJA, van Lanschot JJB, Ristimäki A: Prognostic significance of elevated cyclooxygenase-2 expression in patients with adenocarcinoma of the esophagus. Gastroenterology, 2002;122:1800-1807.

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ABBREVIATIONS

AA	arachidonic acid
ACF	aberrant crypt foci
Apc	adenomatous polyposis gene
cDNA	complementary deoxyribonucleic acid
COX	cyclooxygenase
DDFS	distant disease-free survival
DCIS	ductal carcinoma *in situ*
DMBA	7,12-dimethylbenz(a)anthracene
ER	estrogen receptor
FAP	familial adenomatous polyposis
IL	interleukin
Min	multiple intestinal neoplasia
mRNA	messenger ribonucleic acid
MMP	matrix metalloproteinase
NSAID	nonsteroidal anti-inflammatory drug
PG	prostaglandin (e.g., PGD_2, PGE_2, $PGF_{2\alpha}$, PGG_2, PGH_2, PGI_2)
PR	progesterone receptor
PhIP	2-amino-1-methyl-6-phenylimid-azo[4,5-b]pyridine
PLA_2	phospholipase A_2
PPAR	peroxisome proliferator-activated receptor
RT-PCR	reverse transcriptase polymerase chain reaction
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

ABSTRACT

Epidemiological studies indicate that use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with reduced risk for cancer of the digestive tract including esophageal carcinoma. Association of NSAID use with incidence of non-gastrointestinal malignancies is less clear, but a recent report showed that regular use of aspirin reduced risk for breast cancer especially in hormone receptor-positive patients. The best-known target of NSAIDs is the cyclooxygenase (COX) enzyme. Two isoforms of COX are known. COX-1 is expressed in a constitutive manner, with its role connected to physiological functions such as cytoprotection of the stomach and control of platelet aggregation. In contrast, expression of COX-2 is undetectable in most healthy tissues, but can be induced in response to cell activation by proinflammatory cytokines, growth factors, and tumor promoters, and its role has been connected to inflammation and carcinogenesis. Functionally, COX-2-derived prostanoids inhibit apoptosis, promote angiogenesis, induce invasion, and increase metastasis. Genetic deletion of COX-2 in mouse models of familial adenomatous polyposis (FAP) leads to a reduced polyp burden, and in a transgenic mouse model, overexpression of COX-2 is sufficient to promote mammary gland tumorigenesis. In addition, two different selective COX-2 inhibitors have reduced polyp burden in patients with FAP. In breast and esophageal cancer patients, expression of COX-2 is elevated, but the clinical relevance of this finding is yet unknown.

The purpose of this study was to investigate whether expression of COX-2 is associated with clinicopathological parameters and clinical outcome in human breast and esophageal cancer patients. In breast cancer specimens, we also investigated the correlation of COX-2 expression with HER-2, estrogen receptor (ER), progesterone receptor (PR), p53, and matrix metalloproteinase-2 (MMP-2). In respect to esophageal cancer, we evaluated COX-2 protein expression and prognostic significance in two different histological types of esophageal carcinoma: adenocarcinoma and squamous cell carcinoma. In addition, we investigated whether the prognostic significance of COX-2 depends on patients' preoperative chemotherapy.

Our results show that expression of COX-2 protein was associated with decreased survival in two independent cohorts of breast cancer patients. Interestingly, our data indicate that the prognostic value of COX-2 expression tended to be more marked in certain subgroups of patients, e.g., those with estrogen receptor positive tumors. Thus, the tumor-promoting effect of COX-2 may not be evenly distributed throughout a breast cancer cohort, and certain subgroups of patients may benefit more from COX-2-targeted therapy. In order to understand the carcinogenic mechanism of COX-2 in human malignancies, we correlated its expression with expression of MMP-2, known to promote cancer cell invasion and metastasis. Our data show that expression of COX-2 was associated with MMP-2 expression in breast carcinoma specimens. This suggests that COX-2-derived prostanoids may induce expression of MMP-2 in human tumors, which has previously been shown in experimental cancer models.

Our study was the first to report that in adenocarcinoma of the esophagus, high expression of COX-2 is associated with reduced survival. In contrast, in esophageal squamous cell carcinoma, COX-2 expression was not associated with patient outcome. Low COX-2 expression was, however, associated with poor prognosis in patients with esophageal squamous cell carcinomas who received neoadjuvant chemotherapy. Taken together, our results suggest that the prognostic significance of COX-2 differs depending on the histological type of esophageal carcinoma and on the treatment applied.

REVIEW OF THE LITERATURE

COX and cancer

COX-1 and COX-2 enzymes

For thousands of years, one treatment for pain has been willow bark. Its active constituent was found to be salicylate, and its better-tolerated derivate acetylsalicylate was introduced as aspirin by the middle of the nineteenth century. In 1971 its mechanism of action was discovered when Vane found that aspirin blocks the production of prostaglandins (PG) (Vane, 1971). Vane and two Swedish scientists, Bergström and Samuelsson, won the Nobel Prize in Physiology and Medicine for that discovery in 1982. COX enzyme, now known as COX-1, was first characterized from bull vesicular glands in 1976 (Miyamoto *et al.*, 1976). Three groups cloned COX-1 cDNA from sheep seminal vesicles in 1988 (DeWitt and Smith, 1988; Merlie *et al.*, 1988; Yokoyama *et al.*, 1988), followed by cloning of human cDNA from the platelets in 1991 (Funk *et al.*, 1991). It soon became evident that there might exist several isoforms of the COX enzyme: constitutive and inducible. In 1991, two independent groups reported simultaneously an inducible COX enzyme called COX-2 (Kujubu *et al.*, 1991; Xie *et al.*, 1991), and in 1992, human COX-2 cDNA was cloned from human umbilical vein endothelial cells (Hla and Neilson, 1992).

COX-1 is expressed constitutively in most tissues and cells except red blood cells. It is believed to be primarily a housekeeping gene responsible for synthesis of the PGs that control normal physiological functions such as maintenance of the gastric mucosa, platelet aggregation, and regulation of renal blood flow. On the other hand, COX-2 is an inducible immediate-early response gene undetectable in most normal tissues but induced by various cytokines, growth factors, hormones, and tumor promoters (Herschman, 1996). At cellular level, both COX enzymes are expressed in the endoplasmic reticulum and nuclear envelope (Morita *et al.*, 1995; Spencer *et al.*, 1998) (Table 1). A new COX variant recently identified and designated COX-3 has the same structure as COX-1, except that its mRNA has retained intron-1, which is also translated into a 30-amino acid

extension to the protein (Chandrasekharan *et al*., 2002). Thus, it is a splicing variant of COX-1 (Chandrasekharan and Simmons, 2004).

Table 1: Properties of human COX-1 and COX-2 genes and gene products.

Property	**COX-1**	**COX-2**
Chromosomal location	Chromosome 9	Chromosome 1
Gene size	22 kb	8.3 kb
Number of exons	11	10
Number of introns	10	9
mRNA size	2.7 kb	4.5 kb
Regulation	Constitutive	Inducible
Molecular weight	72 kDa	72 kDa
Subcellular location	Endoplasmic reticulum and nuclear envelope	Endoplasmic reticulum and nuclear envelope

COX enzymes, which catalyze the rate-limiting step in PG synthesis, the conversion of arachidonic acid (AA) (a 20-carbon polyunsaturated fatty acid) to PGH_2, consist of three domains: an epidermal growth factor domain, a membrane-binding motif, and an enzymatic domain containing both COX and peroxidase activity sites (Luong *et al*., 1996). The first step in PG synthesis is mobilization of AA from membrane phospholipids in a reaction catalyzed by the phospholipase A_2 (PLA_2) family of enzymes. The next step is catalyzed by COX, which inserts molecular oxygen into AA. This reaction produces an unstable product, PGG_2, which is rapidly converted by COX peroxidase activity to PGH_2. Specific synthases then convert PGH_2 to various prostanoids, including PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2, each prostanoid having its own range of biological activities. Different PGs are synthesized in a broad range of tissue types and act as autocrine or paracrine mediators to signal changes within the immediate environment (Simmons *et al*., 2004). PGs act through binding to transmembrane, G-protein-coupled cell-surface receptors that act through changes in cellular levels of cyclic AMP and Ca+. In addition, PGs can act directly within the nucleus

through nuclear peroxisome proliferator-activated receptors (PPARs) (Simmons *et al.*, 2004).

Table 2: COX-2 expression in carcinoma.

Adenocarcinoma	
Colorectal	Eberhart *et al.*, 1994
Breast	Parrett *et al.*, 1997
Stomach	Ristimäki *et al.*, 1997
Esophagus	Wilson *et al.*, 1998
Lung	Huang *et al.*, 1998b
Liver	Shiota *et al.*, 1999
Pancreas	Tucker *et al.*, 1999
Cervix	Ryu *et al.*, 2000
Endometrial	Tong *et al.*, 2000
Prostate	Gupta *et al.*, 2000
Ovarian	Matsumoto *et al.*, 2001
Thyroid	Specht *et al.*, 2002
Squamous cell carcinoma	
Lung	Huang *et al.*, 1998b
Skin	Buckman *et al.*, 1998
Esophagus	Zimmermann *et al.*, 1999
Head & neck	Chan *et al.*, 1999

COX-2 expression in cancer

While COX-2 expression is undetectable in most normal tissues, it is overexpressed in several different types of carcinoma. Eberhart *et al.* (1994) were the first to document elevated expression of COX-2 in human colorectal carcinoma and adenoma. Other studies have confirmed that COX-2 mRNA and protein are elevated in 75 to 100% of human colorectal cancers (Kargman *et al.*, 1995; Sano *et al.*, 1995; Kutchera *et al.*, 1996). Similar to colorectal cancer, COX-2 is expressed in several other types of carcinomas, including breast and esophagus (Table 2). COX-1 expression can be detected in most tissues and cells

except red blood cells. In contrast to COX-2, the level of COX-1 expression is not usually elevated in malignant tissues when compared to levels in nonneoplastic tissue of the same organ (Dubois *et al.*, 1998).

NSAIDs and cancer

One of the first clues that COX inhibition might be an effective means to prevent cancer came from epidemiological studies. Epidemiological data indicate that risk for colorectal cancer is reduced by approximately half in those regularly taking aspirin or other NSAIDs (Thun *et al.*, 1991; Thun *et al.*, 1993; Giovannucci *et al.*, 1994; Giovannucci *et al.*, 1995). Since NSAIDs are known to inhibit COX enzyme activity, elevated prostaglandin levels may contribute to development of colorectal cancer. In addition, epidemiological data suggest that NSAIDs may also reduce risk for cancers in the stomach (Langman *et al.*, 2000), esophagus (Table 3), and breast (Khuder and Mutgi, 2001; Terry *et al.*, 2004).

Gastrointestinal side-effects of NSAIDs such as ulceration, bleeding, and perforation are presumably mediated via inhibition of COX-1. Therefore, selective COX-2 inhibitors were created to avoid NSAID-related gastrointestinal side-effects. That the substrate-binding site of COX-2 is larger and has a slightly different shape than that of COX-1 allows selective COX-2 inhibitors to bind in this additional space (Luong *et al.*, 1996). Long-term use of selective COX-2 inhibitors has now been tested in several large randomized trials (Langman *et al.*, 1999; Simon *et al.*, 1999; Silverstein *et al.*, 2000), confirming their improved gastrointestinal safety profile compared to that of non-selective COX-2 inhibitors. Concern has, however, been raised as to the possible cardiovascular risk associated with these new drugs (Mukherjee *et al.*, 2001). Indeed, it was reported recently that three different COX-2 inhibitors: rofecoxib, celecoxib, and valdecoxib all increased cardiovascular toxicity in three large, randomized, controlled trials (Bresalier *et al.*, 2005; Nussmeier *et al.*, 2005; Solomon *et al.*, 2005)

Table 3: Retrospective studies on the protective effect of NSAIDs for esophageal adenocarcinoma.

Reference	Method	Sample	Outcome	NSAIDs results
Thun *et al.*, 1993	Epidemiologic study Follow-up	n = 635 031	Death rates	RR = 0.59 95% CI = 0.34-1.03
Funkhouser and Sharp, 1995	Epidemiologic study Follow-up	n = 14 407	Esophageal cancer incidence	RR = 0.10 95% CI = 0.01-0.76
Farrow *et al.*, 1998	Case control Population-based	Cases: 650 Controls: 695	Esophageal cancer incidence	OR = 0.37 95% CI = 0.24-0.58
Langman *et al.*, 2000	Case control Population-based	Cases: 12 174 Controls: 34 934	Esophageal cancer incidence	OR = 0.64 95% CI = 0.41-0.98
Coogan *et al.*, 2000	Case control Hospital based	Cases: 1 149 Controls: 5 952	Esophageal cancer incidence	OR = 0.8 95% CI = 0.5-1.4

RR = relative risk; CI = Confidence Interval; OR = Odds Ratio.

In addition to inhibiting tumor growth via COX inhibition, NSAIDs can act through COX-independent mechanisms, e.g., by antagonizing the NF-κB signaling pathway (Kopp and Ghosh, 1994), by reducing levels of the antiapoptotic protein Bcl-X_L (Zhang *et al.*, 2000), or through PPARs (Lehmann *et al.*, 1997; He *et al.*, 1999). However, concentrations of the drugs required to affect these pathways are relatively high; at pharmacological doses, COX enzymes are the best-characterized targets of NSAIDs (Gupta and Dubois, 2001).

Animal studies

The association between COX-2 and tumorigenesis has been evaluated in various rodent models. In an azoxymethane-induced rat model of colon carcinogenesis, animals develop pre-neoplastic colonic lesions called aberrant crypt foci (ACF),

which later progress into carcinomas. Aspirin and the COX-2 selective inhibitor celecoxib reduced ACF formation and multiplicity (Reddy *et al*., 1993; Kawamori *et al*., 1998; Reddy *et al*., 2000). In a nude mouse xenograft model, both the non-selective COX-inhibitor meloxicam (Goldman *et al*., 1998) and the selective COX-2 inhibitors celecoxib and SC-58125 (Sheng *et al*., 1997; Williams *et al*., 2000b) reduced colon carcinoma growth. In addition, SC-58125 could reduce tumor formation in nude mice in a COX-2-expressing cell line, whereas no effect appeared with a non-COX-2-expressing cell line (Sheng *et al*., 1997). *Min* mice (Su *et al*., 1992) and $Apc^{\Delta 716}$ mice (Oshima *et al*., 1995) develop multiple adenomas predominantly in the small intestine (Bilger *et al*., 1996). Both non-selective NSAIDs and selective COX-2 inhibitors can reduce polyp formation in these mice (Boolbol *et al*., 1996; Oshima *et al*., 2001). In addition to prevention studies, COX-2 inhibitors are also being evaluated as therapeutic agents for pre-existing tumors; Celecoxib reduces tumor multiplicity in *Min* mice when administered after adenomas have been established (Jacoby *et al*., 2000).

More strikingly, genetic disruption of COX-2 inhibits polyp size and number in $Apc^{\Delta 716}$-knockout mice, and the polyp number fell by 66% in *Cox-2*+/- mice and 86% in *Cox-2*-/- mice, making the effect gene-dose dependent (Oshima *et al*., 1996). This provides direct genetic evidence that COX-2 plays a role in intestinal polyp formation. However, one study showed an equivalent reduction in tumor multiplicity in *Min* mice that were null for either *Cox-1* or *Cox-2* (Chulada *et al*., 2000). A study by Liu *et al*. (2001) was the first to show that overexpression of *COX-2* alone is sufficient to induce cellular transformation. They created a transgenic mouse that expresses the human *COX-2* gene under the MMTV promoter, which directs the expression in the mammary glands. These mice, after multiple pregnancies, develop mammary gland hyperplasias, dysplasias, and metastatic tumors.

Clinical evidence

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited disease, in which patients develop numerous adenomatous polyps in the colon

that predispose to colorectal carcinoma. The genetic mutation responsible for this disease is a germline mutation of the *APC* gene. Somatic mutations in the *APC* gene occur also in a large number of sporadic colorectal cancers. A randomized, double blind, placebo-controlled clinical trial showed that sulindac, a non-selective COX inhibitor, can reduce size and number of colonic polyps in FAP patients (Giardiello *et al*., 2002). In addition, recently two different selective COX-2 inhibitors reduced polyp burden in patients with FAP. In a randomized, double-blind, placebo-controlled study with 77 FAP patients, Steinbach *et al*. (2000) showed that treatment twice daily with 400 mg celecoxib caused a 28% reduction in the number of colorectal polyps compared with a 4.5% reduction for placebo. Polyp burden was also reduced in the celecoxib group. Higuchi *et al*. (2003) obtained similar results with another selective COX-2 inhibitor, rofecoxib. The promising results of these FAP studies have led to several ongoing clinical trials of selective COX-2 inhibitors (Anderson *et al*., 2002).

Role of COX-2 in tumorigenesis

During tumorigenesis, increased COX-2 expression is likely to be a consequence of multiple effects. For example, transcriptional activation can occur in response to growth factors and oncogenes. Several oncogenes that can upregulate COX-2 expression include *v-src*, *v-Ha-ras*, *HER-2*, and *Wnt* genes (Howe *et al*., 2001). COX-2 expression can also be controlled by regulating COX-2 mRNA stability (Ristimäki *et al*., 1994; Dixon *et al*., 2000). The COX-2 product PGE_2 is thought to be responsible for the cancer-promoting effects of COX-2. A recent study demonstrated that PGE_2 could mediate its action by transactivation of epidermal growth factor receptor in gastric epithelial cells, colon cancer cells, and in rat gastric mucosa (Pai *et al*., 2002). Furthermore, mice lacking the PGE_2 receptor subtypes EP_1 or EP_4 showed a decreased formation of chemically induced ACF (Watanabe *et al*., 1999; Mutoh *et al*., 2002), and genetic disruption of the EP_2 receptor reduced the number and size of intestinal polyps in $Apc^{\Delta 716}$ mice (Sonoshita *et al*., 2001). Synthesis of PGE_2 seems to be mechanistically linked to formation of neoplasias by its inhibiting apoptosis, promoting angiogenesis, inducing metastasis and invasion, and inducing immunosuppression (Figure 1).

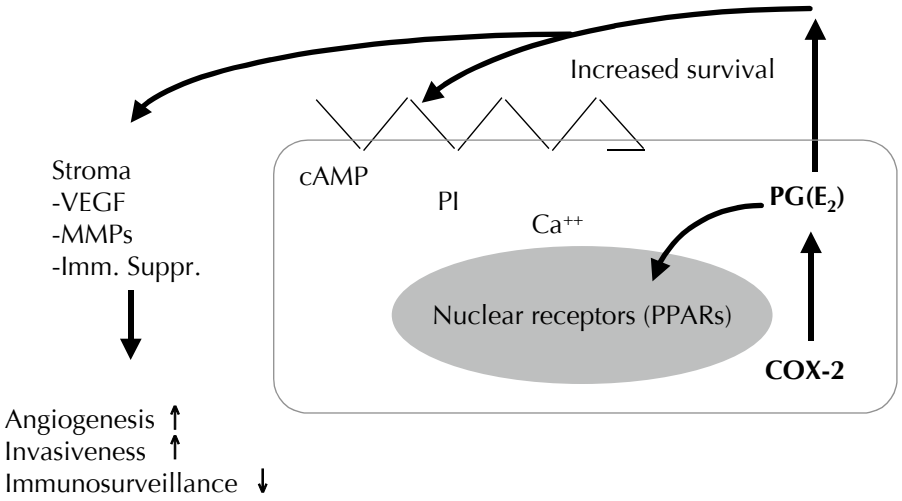

Figure 1: Putative roles of COX-2 in carcinogenesis.

Tumor growth depends on a balance between cell proliferation and apoptosis (Hanahan and Weinberg, 2000). Several studies demonstrate a connection between apoptotic response and COX-2 expression. Overexpression of COX-2 in rat intestinal epithelial cells has elevated amounts of the anti-apoptotic Bcl-2 protein and led to resistance to butyrate-stimulated apoptosis (Tsujii and DuBois, 1995). In colorectal cancer cells, PGE_2 can upregulate Bcl-2 protein and suppress apoptosis induced by a selective COX-2 inhibitor (Sheng *et al.*, 1998). Celecoxib can induce apoptosis in human prostate cancer cells (Hsu *et al.*, 2000), and the COX-2 inhibitior NS-398 can induce apoptosis through a cytochrome c-dependent pathway in esophageal cancer cells (Li *et al.*, 2001a). In COX-2 transgenic mice, the tumor tissue expresses reduced levels of the proapoptotic proteins Bax and Bcl-x_L. In addition, mammary gland involution is delayed, with a decrease in the apoptotic index of mammary epithelial cells. Alternatively, since AA stimulates cells to undergo apoptosis, enhanced COX-2 expression, by increasing the conversion of AA to PGs, can inhibit apoptosis (Cao *et al.*, 2000).

COX-2 can also promote tumorigenesis through direct action on the stromal compartment by stimulating development of tumor-associated angiogenesis. This is an important step in tumorigenesis, since neovascularisation is necessary for

tumors to grow beyond 3 mm in size (Folkman, 2002). Overexpression of COX-2 in colon cancer cells raises the production of angiogenic factors and formation of capillary-like networks; the selective COX-2 inhibitor NS-398 can inhibit these effects (Tsujii *et al*., 1998). Recently, a study showed that PGE_2 and PGI_2 have an essential function in endothelial cell spreading and migration mediated by the integrin αVβ3 (Dormond *et al*., 2001). In addition, COX-2-overexpressing cells can up-regulate vascular endothelial growth factor (VEGF)-C and lymphangiogenesis in human lung adenocarcinoma cells (Su *et al*., 2004). *In vivo*, corneal blood vessel formation is suppressed by celecoxib, but not by the COX-1 inhibitor SC-560, in a rat model of angiogenesis (Masferrer *et al*., 2000). Interestingly, host COX-2 is important in tumor neovascularization, because Lewis lung carcinoma xenocrafts, implanted in COX-2 knockout mice, showed reduction in growth and vascular density when compared to tumors implanted in wild-type mice. Consistently, COX-2-null fibroblasts showed reduced ability to produce VEGF compared to that of the wild-type (Williams *et al*., 2000a). Indeed, COX-2 expression is not restricted to the epithelial component of the human tumor, since endothelial cells of the angiogenic vasculature in human colon, breast, prostate, and lung cancer biopsy tissues also expresses COX-2 (Masferrer *et al*., 2000). In addition, in human breast tumors, COX-2 expression correlates with staining of CD31, an endothelial cell marker of angiogenesis (Davies *et al*., 2003).

COX-2 has been shown to induce invasion and metastasis. In a Matrigel invasion assay, overexpression of COX-2 in colorectal cancer cells can enhance invasiveness and induce expression or activity of several enzymes capable of digesting the basement membrane (Tsujii *et al*., 1997). In non-small cell lung carcinoma cells, stable overexpression of COX-2 induced invasiveness of the cells (Dohadwala *et al*., 2002), and in prostate cancer cells, the COX-2 selective inhibitor NS-398 suppressed invasiveness (Attiga *et al*., 2000). In addition, adding PGE_2 to colorectal cancer cells leads to an increase in cell proliferation and motility associated with activation of the PI3K/AKT pathway (Sheng *et al*., 2001). In animal models, selective blockade of COX-2 suppresses metastasis. The COX-2

selective inhibitor JTE-522 reduced the amount of lung metastasis in mice injected with COX-2-overexpressing colorectal cancer cells, but this was not the case with low COX-2-expressing cells (Tomozawa *et al*., 1999). In addition, a recent study showed that the COX-2 selective inhibitor rofecoxib could reduce metastasis in a mouse model of colorectal carcinogenesis (Yao *et al*., 2003).

COX-2 or PGs can regulate host immune surveillance. PG-mediated immunosuppression may contribute to tumorigenesis, since it allows tumors to avoid immune surveillance that would otherwise limit cancer growth. This was evident in a murine lung tumor model where inhibition of COX-2 altered the balance between interleukin (IL)-10 and IL-12 (Stolina *et al*., 2000). In addition, PGE_2 can inhibit production of tumor necrosis factor (TNF)α while inducing IL-10 production (Huang *et al*., 1998a).

In addition to its PG-mediated contribution to tumorigenesis, COX-2 overexpression may have PG-independent consequences. COX-2 overexpression may result in increased production of mutagens. Peroxidase activity of COX-2 catalyzes the conversion of procarcinogens, such as benzo[a]pyrene, to carcinogens. This activity is especially important in organs exposed to tobacco carcinogens (Wiese *et al*., 2001). COX-2 can also be induced by procarcinogens present in such substances as tobacco smoke and grilled foods (Kelley *et al*., 1997). Furthermore, by-products of the oxidation of AA such as malondialdehyde are highly reactive and form adducts with DNA (Plastaras *et al*., 2000).

Prognostic role of COX-2 in digestive tract carcinomas

Sheehan *et al.* (1999) were the first to show that expression of COX-2 is associated with tumor aggressiveness. They showed in 76 colorectal cancer patients that extent of COX-2 staining correlated with lymph node involvement, large tumor size, more advanced Dukes stage, and a poorer prognosis. In a more recent study in 100 colon cancer patients, COX-2 protein expression correlated with unfavorable pathological variables, microvessel density, and shorter survival time (Masunaga *et al*., 2000). Neither of these studies demonstrated COX-2 as an

independent prognostic factor. In another study, in 63 colon cancer patients, COX-2 protein overexpression was related to degree of metastasis and local recurrence (Tomozawa *et al*., 2000). In contrast, no correlation appeared between COX-2 expression and survival in 139 colorectal cancer patients (Wu *et al*., 2003). In gastric cancer, COX-2 protein expression was associated with poor prognosis in 71 gastric cancer adenocarcinoma specimens (Chen *et al*., 2001). In contrast, a study on 104 human gastric carcinoma samples found no association between COX-2 protein expression and patient survival (Lim *et al*., 2000). In squamous cell carcinoma of the oral cavity, COX-2 overexpression both at the time of surgery and in recurrences associated with lymph node involvement and poor survival and was, furthermore, an independent predictor for disease-free survival (Itoh *et al*., 2003).

COX-2 and breast cancer

In the Western world, breast cancer is the most common cancer in women, although in recent years lung cancer has surpassed breast cancer as their leading cause of cancer death (Jemal *et al*., 2002). Although epidemiological studies indicate that use of NSAIDs is associated with reduced risk for cancers of the digestive tract (Thun, 1994), association of NSAIDs with breast cancer incidence is less clear. Early studies found a lower incidence of breast cancer in patients with rheumatoid arthritis taking NSAIDs for pain relief (Baron, 1995). Most epidemiological studies have reported an at least 20% to 40% reduction in breast cancer risk with regular use of aspirin or other NSAIDs (Schreinemachers and Everson, 1994; Harris *et al*., 1995; Harris *et al*., 1996; Harris *et al*., 1999; Neugut *et al*., 1998; Sharpe *et al*., 2000; Johnson *et al*., 2002; Harris *et al*., 2003; Kim *et al*., 2003; Garcia Rodriguez and Gonzalez-Perez, 2004;), while other studies have found no association (Egan *et al*., 1996; Coogan *et al*., 1999; Langman *et al*., 2000). The data collected and questions asked varied between the studies, and other NSAID use besides aspirin was not completely reported or not separated from aspirin use. A recent meta-analysis suggests, however, that NSAIDs may reduce risk for breast cancer (Khuder and Mutgi, 2001). Its combined results from six cohort studies and eight case-control studies gave a combined estimate of a

reduced relative risk of 0.82 (95% confidence interval 0.75-0.89). Interestingly, one of these reports showed that regular aspirin use reduced risk for breast cancer especially in hormone-receptor-positive patients (Terry *et al.*, 2004).

COX-2 expression in breast cancer

The role of the PG pathway in breast cancer was first indicated by reports of elevated PG levels in breast carcinomas (Bennett *et al.*, 1977). A high concentration of PGE_2 was associated with development of metastasis, lack of hormone receptors, and poor survival (Rolland *et al.*, 1980). Early studies of COX-2 mRNA and protein expression in invasive breast cancer yielded inconsistent findings, with expression reported in between 0% and 100% of samples. COX-2 expression was detected by RT-PCR in all 13 breast cancers studied, but was not detected in normal tissue specimens, and immunohistochemistry revealed COX-2 protein in tumor cells but not in stromal cells (Parrett *et al.*, 1997). No COX-2 expression was found in normal breast tissue specimens, but COX-2 mRNA expression was detected in all 21 cancer samples (Brueggemeier *et al.*, 1999). In contrast, COX-1 was elevated in the stroma in 30 of 44 samples studied by Western blotting, but COX-2 was elevated in only 2 of 44 of these tumors (Hwang *et al.*, 1998). Immunohistochemical studies of COX-2 expression have produced more consistent findings, with high COX-2 expression in 17 to 56% of invasive breast cancers (Ratnasinghe *et al.*, 2001). Soslow *et al.* (2000) found immunohistochemical evidence of moderate to strong levels of COX-2 expression in 41% of invasive breast cancer and in 80% of ductal carcinoma *in situ* (DCIS) surrounding the invasive cancer. Consistent with this, several other studies have reported higher COX-2 expression in DCIS than in invasive breast carcinomas (Half *et al.*, 2002; Watanabe *et al.*, 2003; Boland *et al.*, 2004b). Furthermore, a recent report suggested that COX-2 is upregulated in the DCIS but is even more upregulated in normal adjacent epithelium (Shim *et al.*, 2003), which suggests the hypothesis that in DCIS the surrounding epithelial tissue is part of the disease process. The same group later reported that in normal human breast epithelium, overexpression of COX-2 coincides with focal areas of INK4a hypermethylation

(Crawford *et al*., 2004). They argued further that these focal areas might represent early neoplastic changes later leading to breast cancer.

Animal studies

COX-2 protein has been detected in carcinogen-induced rat models of mammary tumorigenesis (Nakatsugi *et al*., 2000). Levels of COX-2 protein are elevated in mammary tumors of MMTV/neu transgenic mice (Howe *et al*., 2002), and COX-2 is transcriptionally upregulated in *Wnt-1* expressing mouse mammary epithelial cell lines (Howe *et al*., 1999). In addition, mammary epithelial cells transformed by *src* or *ras* oncogenes produce increased amounts of COX-2 mRNA and PGE_2 (Subbaramaiah *et al*., 1996).

Carcinogen-induced rat mammary tumors have served as a model system to test various NSAIDs and selective COX-2 inhibitors as chemopreventive agents. Ibuprofein reduces tumor volume in 7,12-dimethylbenz(a)anthracene (DMBA) -induced rat mammary carcinomas (Robertson *et al*., 1998), with similar results in studies using indomethasin (McCormick *et al*., 1985; Lala *et al*., 1997). Several recent studies have evaluated the effects of selective COX-2 inhibitors on mammary tumorigenesis in rats. Harris *et al*. (2000), studying the effect of celecoxib against DMBA-induced mammary carcinogenesis, found it to reduce incidence, multiplicity, and volume of mammary tumors. Another COX-2 selective inhibitor, nimesulide, reduced incidence, size, and multiplicity of 2-amino-1-methyl-6-phenylimid-azo[4,5-b]pyridine (PhIP) -induced rat mammary tumors (Nakatsugi *et al*., 2000). Furthermore, the chemopreventive effect of celecoxib is dose dependent, with increasing doses of inhibitor resulting in decreasing tumor incidence, multiplicity, and volume (Abou-Issa *et al*., 2001). In addition, selective COX-2 inhibitors may play a role in the treatment of established breast cancer, since rats treated with celecoxib after DMBA had a diminished tumor volume and number (Alshafie *et al*., 2000). In addition to COX-2, COX-1 may also play a role in mammary carcinogenesis, since Kundu and Fulton (2002) reported that in mice COX-2 as well as COX-1 selective inhibitors can inhibit the growth and metastatic capacity of mammary tumors.

Inhibition of COX-2 has reduced growth of the primary tumor and number and incidence of spontaneous metastases in an orthotopic model of breast cancer (Connolly *et al.*, 2002), accompanied by increased apoptosis and decreased microvessel density in the primary tumor. Similarly, after excision of the primary tumor, COX-2 inhibitor treatment has significantly reduced burden, number, and size of spontaneous lung metastases (Roche-Nagle *et al.*, 2004), with these reductions accompanied by increased apoptosis and decreased microvessel density in the pulmonary metastases. Apparently, COX-2 inhibition therefore not only reduces metastasis by reducing the growth of the primary tumor but also inhibits metastasis. Most strikingly, a recent study demonstrated that expression of COX-2 under an MMTV promoter in the mammary glands is sufficient to induce mammary tumors in transgenic mice (Liu *et al.*, 2001). Virgin females did not develop tumors, but more than 85% of the multiparous mice did, and MMTV-driven COX-2 expression increased during pregnancy, suggesting a basis for the failure of the virgin animals to develop tumors. The same group reported that PGE_2 induces tumor-associated angiogenesis, and this is required for the initiation or progression of mammary cancers or both in MMTV-COX-2 mice. Furthermore, this induction was already evident at the earliest stage of tumor development, even prior to mammary gland hyperplasia (Chang *et al.*, 2004).

COX-2 related factors in breast cancer

HER-2

HER-2 (also known as ErbB2) is overexpressed or amplified or both in a number of human cancers, including breast cancer (Hynes and Stern, 1994). It is overexpressed in approximately a third of the primary breast carcinomas, and this overexpression is associated with decreased survival (Slamon *et al.*, 1987). No ligand has been shown to bind directly to HER-2, but it is activated upon ligand binding to other HER receptors. HER-2 then is thought to function as a coreceptor for other members of the HER family.

Several studies have shown a connection between COX-2 and HER-2 genes. In colorectal cancer cells, activation of HER-2/HER-3 heterodimers by heregulin induces COX-2 promoter activity, mRNA and protein expression, and production of PGE_2 (Vadlamudi *et al.*, 1999). HER-2 expression can, when transfected to breast carcinoma cell lines, induce expression of COX-2 through ras/raf/MAPK signaling and enhanced AP-1 binding (Subbaramaiah *et al.*, 2002). Transfection of HER-2 to breast cancer cells enhances COX-2 gene transcription (Kiguchi *et al.*, 2001) through the Ras/MAPK/AP-1 pathway (Subbaramaiah *et al.*, 2002) or through the Akt pathway (Simeone *et al.*, 2004). COX-2 is overexpressed in the tumors of MMTV/neu transgenic mice, and celecoxib reduces the incidence of mammary tumors in this model (Howe *et al.*, 2002).

In clinical breast cancer samples, Subbaramaiah *et al.* (2002) found high levels of COX-2 protein in 14 of 15 microdissected HER-2-positive breast cancer samples. This was in contrast to the 14 HER-2-negative tumors, with COX-2 expressed in only 4 of the cases. In addition, nuclear HER-2 can associate with a specific sequence in the COX-2 promoter and upregulate expression of the COX-2 gene (Wang *et al.*, 2004). Finally, recent data suggest a regulatory loop between these two genes, since COX-2-derived PGE_2 can regulate HER-2 gene expression in breast cancer cells (Benoit *et al.*, 2004).

Aromatase

Aromatase (estrogen synthetase) is a cytochrome P450 enzyme complex that converts androgens to estrogens. Approximately two-thirds of breast cancers show aromatase activity (Silva *et al.*, 1989), which results in locally high levels of estrogen production that can lead to stimulation of tumor proliferation. PGE_2 can stimulate aromatase expression both in the tumor itself and in the surrounding adipose tissue (Figure 2). In adipose tissue, aromatase is normally expressed from promoter I.4, whereas in adipose tissue adjacent to breast tumors, the aromatase gene *CYP19* is expressed from promoter II. PGE_2 elevates intracellular cAMP levels and stimulates aromatase expression by activating the promoter II region of the *CYP19* gene in breast adipose stromal cells (Zhao *et al.*, 1996). In human

breast cancer samples, expression of aromatase and COX-2 correlates in mRNA and protein levels (Brodie *et al.*, 2001; Brueggemeier *et al.*, 1999).

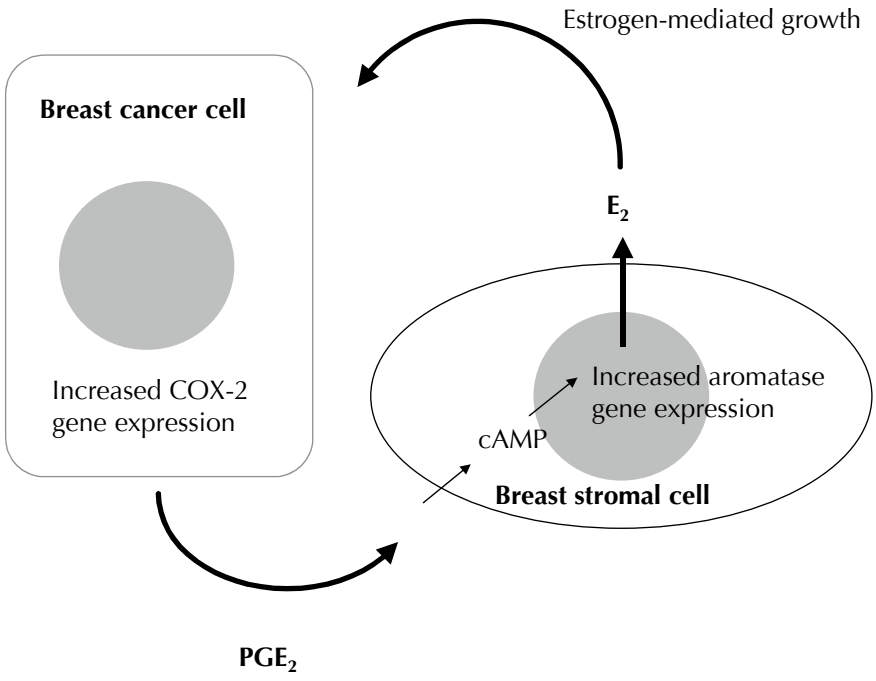

Figure 2: Prostaglandin produced by breast cancer cells induces expression of aromatase in breast stromal cells.

Aromatase inhibitors are currently under evaluation for their utility in cancer chemoprevention. These drugs, however, suppress estrogen formation throughout the body and may therefore lead to unwanted side-effects, particularly in bone resorption. COX-2 inhibitors could provide a mechanism for selective suppression of estrogen in the breast. Clinical studies of the chemopreventive use of COX-2 inhibitors are underway to test their effect in breast cancer.

Hormone receptors

Approximately one-third of all breast cancer patients and two-thirds of the postmenopausal ones have hormone-dependent breast cancer, which contains estrogen receptors and requires estrogen for tumor growth. Because estrogens bind to specific nuclear receptor proteins where they induce growth and proliferation of breast epithelial cells and estrogen-dependent breast cancer cells, deprivation of estrogen signaling is an important form of therapy for patients with estrogen receptor positive or progesterone receptor positive disease. Thus far,

tamoxifen, an antagonist that blocks the binding of estrogen to its receptor, has been the most widely used. That mortality rate from breast cancer has declined during the last decade is thought to be chiefly due to the use of tamoxifen (Ali and Coombes, 2002). Because tamoxifen can, however, especially during late disease, begin to act as an agonist, aromatase inhibitors have gained attention as inhibitors of estrogen synthesis. Clinical data have shown that aromatase inhibitors are so efficient that they may in the future replace tamoxifen therapy (Johnston and Dowsett, 2003).

Taking into account the fact that PGs, by stimulating aromatase gene expression, stimulate estrogen biosynthesis, the ability of NSAIDs to protect against breast cancer can vary depending on a patient's hormone-receptor status. This hypothesis was tested in a recent study in which regular use of aspirin reduced risk for invasive or *in situ* breast cancer by 20%, and this reduction was more pronounced for women with hormone (estrogen and/or progesterone) receptor-positive tumors (Terry *et al*., 2004). This result is consistent with preclinical and clinical observations that COX-2 can induce aromatase expression and estrogen biosynthesis (Zhao *et al*., 1996; Brueggemeier *et al*., 1999; Brodie *et al*., 2001). Clinical trials are underway on the efficacy of COX-2 inhibitors and aromatase inhibitors in treatment of breast cancer (Chow *et al*., 2003).

p53

p53 is a tumor-suppressor gene involved in the control of cell cycle progression, DNA integrity, and cell survival. Wild-type p53 inhibits COX-2 expression in cell culture (Subbaramaiah *et al*., 1999), so the loss-of-function p53 mutation is able to contribute to COX-2 upregulation. In contrast, COX-2 expression is induced by p53-mediated activation of the Ras/Raf/ERK cascade (Han *et al*., 2002). In clinical studies, higher amounts of COX-2 appeared in gastric cancers containing mutant p53 than in those containing wild-type p53 (Leung *et al*., 2001). Furthermore, in colorectal cancer cells, celecoxib can inhibit wild-type p53 accumulation in the cytosol (Swamy *et al*., 2003). All this raises the possibility that p53 status is one of the determinants of COX-2 expression.

MMP-2

MMP-2 is an extracellular matrix-degrading proteolytic enzyme linked to invasion and metastasis (Liotta *et al.*, 1980; Egeblad and Werb, 2002). Expression and activity of MMP-2 in human breast cancer is elevated, and correlates with lymph-node and distant metastasis, and with shortened survival (Talvensaari-Mattila *et al.*, 2003). A direct link between COX-2 and MMP-2 has been shown in several experimental models. A COX-2 selective inhibitor suppressed the invasiveness and proMMP-2 release of prostate cancer cells (Attiga *et al.*, 2000), and in non-small cell lung carcinoma cells, PGE_2 induced invasion and expression of MMP-2 (Dohadwala *et al.*, 2002). In line with these data, a selective COX-2 inhibitor has been shown to reduce MMP-2 promoter activity, which was partially reversed by administration of PGE_2 (Pan *et al.*, 2001). In addition, transfection of COX-2 elevated the activation of MMP-2 in human breast and colon cancer cells (Tsujii *et al.*, 1997), and a recent study showed in a mouse model of colorectal cancer that the COX-2 selective inhibitor rofecoxib can reduce metastasis and reduce expression of MMP-2 (Yao *et al.*, 2003). In a clinical series, Miyata *et al.* (2003) showed that expression of COX-2 correlates with expression of MMP-2 in renal cell carcinoma and that both of these factors associate with reduced survival.

COX-2 in esophageal carcinoma

Esophageal carcinoma exists in two main forms, adenocarcinoma and squamous cell carcinoma (Figure 3). More than 90% of esophageal cancers worldwide are squamous cell carcinomas, but adenocarcinomas are more prevalent in Western countries, in which incidence of esophageal adenocarcinoma has increased dramatically over the last decades, a rise being faster than for any other solid tumor (Chow *et al.*, 1995; Devesa *et al.*, 1998). The cause for this epidemiological change remains in part unclear. Several risk factors have been proposed; reflux disease (Chow *et al.*, 1995; Cameron and Romero, 2000), smoking (Castellsague *et al.*, 1999), dietary factors (Ribeiro *et al.*, 1996), obesity (Chow *et al.*, 1998b), and changes in *Helicobacter pylori* infection frequencies (Chow *et al.*, 1998a). Reflux disease is clearly a risk factor for adenocarcinoma of the esophagus. A

recent population-based, case-control study found a strong association between symptomatic gastroesophageal reflux disease and risk for esophageal adenocarcinoma (Lagergren *et al.*, 1999). Smoking habits have not changed sufficiently to explain changes in incidence, but obesity has increased during the last decades in the USA and Europe. Indeed, a multicenter population-based case-control study showed that obesity is a strong risk factor for adenocarcinoma of the esophagus (Chow *et al.*, 1998b). Because *Helicobacter pylori* infection apparently protects against Barrett's esophagus and adenocarcinoma (Chow *et al.*, 1998a), the decrease in *Helicobacter pylori* infections may thus explain some of the increase in incidence of esophageal adenocarcinoma. It is now generally accepted that most, if not all, adenocarcinomas of the esophagus develop from a premalignant lesion of the esophagus called Barrett's esophagus (Cameron *et al.*, 1995). In Barrett's esophagus, normal esophageal squamous epithelium in the lower esophagus is replaced by metaplastic columnar intestinal-type epithelium (Spechler and Goyal, 1996) (Figure 3). Patients with Barrett's esophagus are at risk for developing dysplasia leading to adenocarcinoma.

In contrast to esophageal adenocarcinoma, in Western countries the incidence of esophageal squamous cell carcinoma has remained the same or decreased over recent decades (Devesa *et al.*, 1998). Its incidence shows great geographical variation in incidence and mortality. That it occurs at high frequencies in certain parts of the world points to specific environmental factors playing a role in its etiology. In developed countries, its etiology has been mainly attributed to tobacco smoking and heavy alcohol drinking (Castellsague *et al.*, 1999). Consumption of certain foods or hot beverages and microbial toxins has also been connected to its pathogenesis especially in high-incidence areas (Ribeiro *et al.*, 1996). The principal precursor lesion of esophageal squamous cell carcinoma is dysplasia (Figure 3).

Esophageal carcinomas are highly lethal diseases, the overall 5-year survival for patients with advanced disease being less than 20%. Existing treatment modalities are therefore insufficient, and new preventive and treatment strategies are needed.

Among epidemiological studies of esophageal cancer, most have indicated that reduced risk is associated with use of NSAIDs (Funkhouser and Sharp, 1995; Farrow *et al.*, 1998; Langman *et al.*, 2000; Kim *et al.*, 2003). Consistent with this, a recent meta-analysis suggests that NSAIDs are protective against both esophageal adenocarcinoma and squamous cell carcinoma; combining results from two cohort studies and six case-control studies gives a combined estimate of reduced relative risk of 0.67 (95% CI 0.51-0.87) for adenocarcinoma and 0.58 (95% CI 0.43-0.78) for squamous cell carcinoma (Corley *et al.*, 2003).

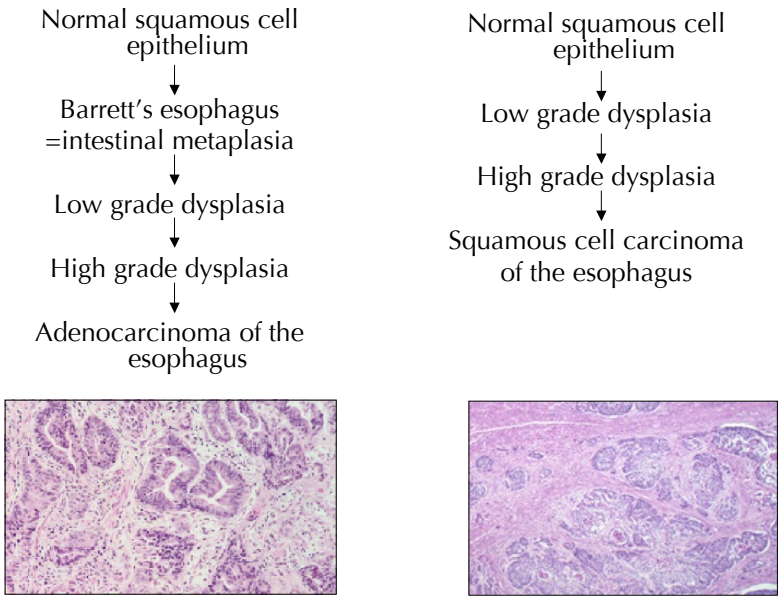

Figure 3: Two types of esophageal carcinoma.

COX-2 expression in esophageal carcinoma

Several studies have reported an elevated COX-2 mRNA expression in Barrett's metaplasias and in associated esophageal adenocarcinoma (Wilson *et al.*, 1998; Shirvani *et al.*, 2000; Lagorce *et al.*, 2003). Moreover, progression from Barrett's esophagus to dysplasia and invasive carcinoma is associated with an increase in COX-2 expression (Wilson *et al.*, 1998; Shirvani *et al.*, 2000). Expression of COX-2 mRNA and protein have also been reported in squamous cell carcinoma of the

esophagus. (Ratnasinghe *et al*., 1999; Shamma *et al*., 2000; Kawabe *et al*., 2002; Kuo *et al*., 2003; Maaser *et al*., 2003). Again, expression of COX-2 increases progressively when nonneoplastic esophageal tissues are compared to dysplastic or to invasive lesions (Shamma *et al*., 2000).

Experimental studies

In vivo and *in vitro* studies both support a possible role for COX-2 in esophageal cancer. Buttar *et al*. (2002a) demonstrated in primary cultured endoscopic biopsy specimens from patients with Barrett's esophagus that the selective COX-2 inhibitor NS-398 reduces COX-2 activity and proliferation of epithelial cells. These same investigators found that in a surgically induced rat reflux model, both the non-selective COX-2 inhibitor sulindac and selective COX-2 inhibitor MF-Tricyclic prevent development of Barrett's esophagus and esophageal adenocarcinoma (Buttar *et al*., 2002b). In a rat model of bile-induced esophageal carcinogenesis, COX-2 expression is induced, and the selective COX-2 inhibitor NS-398 suppresses this expression (Kaur *et al*., 2000). In cell culture, the selective COX-2 inhibitors NS-398 and rofecoxib suppress cellular growth and induce apoptosis in human esophageal adenocarcinoma and squamous cell carcinoma cells (Souza *et al*., 2000; Vona-Davis *et al*., 2004). It is unknown how COX-2 is upregulated in adenocarcinomas of the esophagus, but since bile acids induce COX-2 expression in a rat model (Zhang *et al*., 2001) and in Barrett's explants (Shirvani *et al*., 2000), it is possible that reflux disease may induce the early expression of COX-2 during the development of the Barrett's metaplasia. Finally, Kaur *et al*. (2002) indirectly demonstrated the possible chemopreventive effect of selective COX-2 inhibitors. When biopsy specimens of human Barrett's epithelium were compared with biopsy specimens obtained after 10 days of rofecoxib therapy, a decrease had occurred in COX-2 expression and in PGE_2 content.

In esophageal squamous cell carcinoma cells, both aspirin and NS-398 can induce apoptosis (Li *et al*., 2000; Li *et al*., 2001a). In respect to chemically induced squamous cell tumors in the rat, the data are conflicting, since a COX-2

selective inhibitor has been found to reduce incidence of both noninvasive and invasive lesions (Li *et al*., 2001b), whereas the NSAID piroxicam failed to reduce tumorigenesis (Carlton *et al*., 2002). In a diethylnitrosamine-induced mouse model of esophageal squamous cell carcinoma, NSAID indomethasin is able to reduce tumor incidence (Rubio, 1986). Finally, in a mouse model created for human oral-esophageal cancer, sulindace reduced development of severe dysplasia in the esophagus (Opitz *et al*., 2002).

COX-2 and chemo- and radiotherapy

Results from preclinical and clinical studies suggest a link between COX-2 expression and response to chemo- and radiotherapy treatment. In animal models, the selective COX-2 inhibitors SC-236 and JTE-522 can enhance the efficacy of chemotherapy or radiation treatment (Milas *et al*., 1999; Hida *et al*., 2002). The selective COX-2 inhibitor NS-398 has enhanced the effect of radiotherapy *in vivo* on lung cancer xenocrafts and *in vitro* on human COX-2-overexpressing cells (Pyo *et al*., 2001), and no radiation-enhancing effect occurred in the cells deficient in COX-2 expression. In a rat model of colorectal cancer, celecoxib enhanced the antitumor effect of a chemotherapeutic agent and reduced the side-effect of diarrhea (Trifan *et al*., 2002). Furthermore, the selective COX-2 inhibitor SC-236 does not affect the radiotherapy response of normal tissue (Kishi *et al*., 2000), probably due to the lack of COX-2 expression in normal mouse tissue. Moreover, celecoxib enhances the effects of the chemotherapeutic agent docetaxel and of radiation or of both in nude mice (Nakata *et al*., 2004). In carcinoma cell lines, the selective COX-2 inhibitors nimesulide, SC-236, and NS-398 enhance the radioresponsiveness and cytotoxicity of several chemotherapeutic agents (Hida *et al*., 2000; Petersen *et al*., 2000; Amirghahari *et al*., 2003). Finally, *in vitro* studies and one *in vivo* study show that radiation or chemotherapeutic agents can enhance expression of COX-2 (Steinauer *et al*., 2000; Subbaramaiah *et al*., 2000; Subbaramaiah *et al*., 2003; Davis *et al*., 2004;).

In respect to clinical evidence, Ferrandina *et al*. (2002) demonstrated that in locally advanced cervical cancer patients, receiving neoadjuvant chemotherapy,

COX-2 expression increased progressively from responders to partial responders to non-responders. Furthermore, COX-2-positive patients had a shorter overall survival than did COX-2-negative patients. In oral squamous cell carcinoma patients, low expression of COX-2 is associated with a patient's radiation sensitivity (Terakado *et al.*, 2004). In a very recent study, for esophageal squamous cell carcinoma patients but not for esophageal adenocarcinoma patients, low COX-2 expression, when detected immunohistochemically in pretreatment biopsy specimens, predicted treatment response. Pretreatment COX-2 expression was not, however, associated with their survival (Kulke *et al.*, 2004b). In laryngeal cancer, COX-2 may have prognostic value in predicting response to radiotherapy failure (Nix *et al.*, 2004). Furthermore, in clinical studies of patients with cervical or ovarian cancer receiving adjuvant chemotherapy or radiotherapy, high COX-2 expression associates with resistance to treatment and to reduced overall survival (Gaffney *et al.*, 2001; Kim *et al.*, 2002; Wulfing *et al.*, 2004). Finally, a phase-II trial recently demonstrated that in non-small-cell lung cancer, celecoxib enhances the response to neoadjuvant paclitaxel and carboplatin treatment (Altorki *et al.*, 2003).

AIMS OF THE STUDY

The purpose of this study was to investigate whether expression of COX-2 protein, as detected by immunohistochemistry, is associated with clinicopathological parameters and clinical outcome in human breast and esophageal cancer patients.

The specific aims of the present study were:

- To investigate the prognostic significance of COX-2 in breast carcinoma.

- To correlate COX-2 expression with expression of MMP-2 and with other gene products relevant in breast cancer (HER-2, ER, PR, p53).

- To study the prognostic significance of COX-2 in adenocarcinoma and squamous cell carcinoma of the esophagus.

- To study the prognostic significance of COX-2 in neoadjuvant-treated patients with esophageal squamous cell carcinoma.

MATERIALS AND METHODS

Patients

Breast cancer and esophageal cancer specimens were obtained from several hospitals in Finland and the Netherlands. Information about the patients is in Table 4. All studies were done in accordance with the guidelines of the local ethics committees.

Table 4: Summary of patient samples for this work.

Study	Tumor type	Number of samples	Sample type	Origin
I	Invasive breast cancer	1576	Tissue array	FinProg, Finland
II	Invasive breast cancer	278	Tissue array	Helsinki University Central Hospital, Finland
III	Esophageal adenocarcinoma	145	Histologic	Academic Medical Center, Amsterdam, the Netherlands
IV	Esophageal squamous cell carcinoma	117	Histologic	Academic Medical Center, Amsterdam, the Netherlands

Study I: Five well-defined geographical regions, comprising approximately half the Finnish population, were selected for the study. Using the files of the nation-wide Finnish Cancer Registry, all women diagnosed from 1991 to 1992 with breast cancer were identified. With structured data collection forms, clinical data of 50 characteristics were extracted from hospital records. A total of 2842 patients (93% of all breast cancer patients) with sufficient clinical data available were entered into the database (= the FinProg Breast Cancer Database), of whom 1984 were included the study. Patients with *in situ* carcinomas (n = 201), those with distant metastasis at the time of diagnosis (n = 133), those with synchronous or metachronous bilateral breast cancer (n = 281), those with malignancy other than

breast cancer in their history except for basal cell carcinoma or cervical *in situ* carcinoma (n = 201), and those who underwent no breast surgery (n = 42) were excluded. The median follow-up for patients alive at the end of follow-up was 6.8 years (range 5.1-7.8).

Study II: The patient series comprised 278 other breast cancer cases treated at the Fourth Department of Surgery of the Helsinki University Central Hospital from 1987 to 1990, whenever a histological specimen was available in the pathology files. Onto structured data collection forms, the clinical data of the patients were extracted from the hospital records, which were reviewed. The median follow-up time for patients alive at the end of the study period was 10.1 years (range from 0.3 to 12.6 years).

Studies III and IV: Between 1 January 1993 and 31 December 2000, 306 patients underwent esophageal resection for adenocarcinoma of the esophagus or gastro-esophageal junction, and 504 underwent esophageal resection with proximal gastrectomy for an esophageal malignancy with curative intent (had locally resectable disease without distant metastases) at the Department of Surgery of the Academic Medical Center, Amsterdam, the Netherlands.

For Study III, the data from original 306 patients were prospectively collected in a database. All pathology reports were reviewed to identify those who had undergone surgery for adenocarcinoma developed in a histologically proven Barrett's esophagus, defined by the presence of goblet cells. Patients with an adenocarcinoma of the cardia or gastro-esophageal junction with no Barrett's segment were excluded (n = 155). Archival material from the remaining 151 patients was re-evaluated by two of the investigators to obtain the sample with the deepest invasion of each tumor. Another six specimens were excluded during the immunohistochemical analyses (three samples with no definitive invasive cancer, two with adenosquamous carcinoma, and one that repeatedly detached from the slide), so that only the 145 patients remaining were included. Patients were followed until 30 June 2001 or until death, ensuring a minimal follow-up of 6

months (median 38.2 months; range 8 days to 7.3 years). We included all deaths following surgery, including two perioperative deaths.

For Study IV, the pathology reports of all 504 patients were reviewed to identify 138 patients diagnosed with a squamous cell carcinoma of the esophagus. During review of the slides, 13 tumors with adenosquamous characteristics were excluded, and another 8 patients were excluded during immunohistochemical analyses for having non-representative archival slides compared to the original tumor. Thus, 117 patients were included in the study. Patients were followed until death or December 2003, ensuring a minimal potential follow-up of 3 years. The median actual follow-up was 33.0 months (range 7 days to 8.5 years). No patient was lost to follow-up. Four (3.4%) who died in the hospital within 30 days were included in the analyses.

Neoadjuvant treatment

In Study IV, 36 esophageal squamous cell carcinoma patients received chemotherapy preoperatively as part of a randomized controlled trial: cisplatin (CddP; 80 mg/m^2 iv day 1) and etoposide (E; 100 mg iv day 1+2; 200 mg/m^2 orally days 3+5). After two cycles of chemotherapy, clinical response was evaluated based on clinical findings such as decrease in dysphagia and on repeated endoscopy and in CT scanning. Patients with a tumor response received another two cycles followed by surgery, whereas non-responding patients underwent surgery after the second cycle. No adjuvant treatment was administered postoperatively.

Preparation of breast tumor tissue arrays

Studies I and II incorporated routinely fixed (overnight in 10% buffered formalin) paraffin-embedded tumor samples stored in the files of pathology laboratories. Histopathologically representative tumor regions were defined from hematoxylin- and eosin-stained sections and marked on each slide. Tumor tissue array blocks were made by punching a tissue cylinder (core), diameter 0.6 mm, through a histologically representative area of each ”donor” tumor block, which was then

inserted into an empty ”recipient” tissue array paraffin block with a specialized instrument (Kononen *et al.*, 1998). In Study I, from the 1728 tumor samples available, 19 tissue array blocks were prepared, each containing 50 to 144 tumor samples. For Study II, from the 278 tumor samples available, 4 tissue array blocks were prepared, each containing 36 to 144 tumor samples. Sections of 5 μm were cut and processed for immunohistochemistry. Consecutive sections of each sample were used for COX-2 and MMP-2 immunostaining (II).

Immunohistochemistry

For COX-2 immunostaining (I-IV), formalin-fixed and paraffin-embedded specimens representing the deepest tumor infiltration were sectioned (5 μm), deparaffinized, and microwaved for 4 x 5 min in 700 W in 0.01 M Na-citrate buffer (pH 6.0) for antigen retrieval. The slides were then immersed in 0.6% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity and in blocking solution (1.5:100 normal horse serum in PBS) for 15 min to block unspecific binding sites. Immunostaining was performed with a COX-2-specific mouse anti-human monoclonal antibody (160112, Cayman Chemical Co., Ann Arbor, MI, USA) in a dilution of 1:200 (2.5 μg/ml) in PBS containing 0.1% sodium azide and 0.5% bovine serum albumin at room temperature overnight. Then the sections were treated with biotinylated horse anti-mouse immunoglobulin (1:200; Vector Laboratories Inc., Burlingame, CA, USA) and avidin-biotin peroxidase complex (Vectastain ABComplex, Vector Laboratories). The peroxidase staining was visualized with 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO, USA), and the sections were counterstained with Mayer's hematoxylin. Every 20th sample of the trial series was a known colon adenocarcinoma specimen, in which stromal cells in an area of ulceration were scored 3, cancer cells from 2 to 3, and adjacent nonneoplastic epithelium 1 (for scoring criteria see below). This procedure confirmed that there was no significant intra- and interassay variability in staining intensity, and helped us to score the trial specimens. Recently, we evaluated several COX-2 antibody preparations, and concluded that the monoclonal antibody used in this study provided us with the most specific and reproducible immunoreactivity (Saukkonen *et al.*, 2001).

Antibody specificity was confirmed by staining one breast tumor tissue array slide (I) or a randomly selected subset of esophageal adenocarcinoma specimens (every 10th sample, n = 15) (III) with and without preadsorption of the primary antibody with human COX-2 control peptide (10 µg/ml, Cayman); all cancer cell positivity was blocked by this control procedure.

For MMP-2 immunostaining (II), the breast tissue array specimens were treated with 0.4% pepsin (Sigma) for 20 min at 37°C. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide in absolute methanol for 15 min, and nonspecific binding was blocked with 10% goat serum for 15 min. A mouse monoclonal antibody (CA-4001, Diabor Ltd., Oulu, Finland) to MMP-2 served as the primary antibody (6 µg/ml in 0.01 M phosphate buffer, 0.9% NaCl, pH 7.5), with 1% bovine serum albumin. This antibody recognizes the amino terminal end of the latent MMP-2 both as a free enzyme and when it is in complex with a tissue inhibitor of metalloproteinase-2. Its specificity has been confirmed by a Western blot analysis (Höyhtyä *et al.*, 1994). The specimens were incubated for 60 min at room temperature. The sections were then treated with biotinylated anti-mouse IgG (for COX-2; 1:200, Vector Laboratories, and for MMP-2; Zymed, San Francisco, CA, USA) and the avidin-biotin peroxidase complex was introduced. The peroxidase staining was visualized with 3-amino-9-ethylcarbazole (Sigma), and the sections were counterstained with Mayer's hematoxylin.

For p53 immunostaining (IV), the esophageal squamous cell carcinoma specimens were stained by established procedures (Lundin *et al.*, 1996). Expression of p53, HER-2, ER, and PR (I, II) had previously been determined for breast cancer tissue array datasets. Immunostaining of ER, PR, Ki-67, and p53 was done by established procedures (Järvinen *et al.*, 1998). HER-2 gene amplification had been assessed by chromogenic *in situ* hybridization (Tanner *et al.*, 2001).

Scoring of immunostaining

For evaluating COX-2 immunohistochemical staining, the following scoring criteria for the tumor cells were agreed upon before analysis: 0, no staining; 1+, weak diffuse cytoplasmic staining (may contain stronger intensity in less than 10% of cancer cells); 2+, moderate to strong granular cytoplasmic staining in 10 to 90% of cells; 3+, over 90% stained with strong intensity. Scores 0 and 1 were categorized as "COX-2 low" and scores 2 and 3 as "COX-2 high" for the statistical analyses.

In Study I, COX-2 immunohistochemical staining was scored in breast tissue array samples independently and in a blinded manner by two investigators from 1728 tissue array cores, of which 152 (8.8%) either became detached or contained no tumor cells. The percent agreement between the two independent and blinded investigators in allocation of the tumors into these two categories was 85%. The corresponding kappa value was 0.69, which can be interpreted as a good level of agreement. All specimens with discordant scores were re-evaluated by the two investigators with a multiheaded microscope, and the consensus score was used for further analyses. In Study II, COX-2 immunohistochemical staining was scored for 231 breast tissue array cores (of 278 samples, 20 were detached, and 27 contained no tumor cells). Discordant scores (92% of the specimens were categorized identically) were re-evaluated by the investigators, and this consensus score served for further analysis. COX-2 immunohistochemical staining was evaluated in all esophageal adenocarcinoma and squamous cell carcinoma specimens (III, IV) independently and in a blinded manner by two investigators. Allocation of tumors to the "COX-2 low" versus "COX-2 high" category by the two investigators was similar (> 95% (III), and 93% (IV) of the specimens categorized identically). In cases of disagreement (n = 6 (III) and n = 9 (IV)) the slides were re-evaluated by a group of investigators with a multiheaded microscope.

MMP-2 immunohistochemical staining (II) was scored from 194 tissue array cores (of 278 samples, 26 were detached, and 58 contained no tumor cells). A section was considered negative or positive based on the absence or presence of

cytoplasmic staining; staining was scored as follows: 0, no positive cells; 1, < 20% of the tumor cells staining positive; 2, 20 to 50% staining positive; 3, > 50% positive. Three independent observers scored immunostaining; discordant scores (2%) were re-evaluated by the investigators, with the consensus score used for further analysis.

Immunoreactivity of p53 (IV) was considered weakly positive if 0 to 50% of the tumor cells were immunopositive, and strongly positive if 50 to 100% of the tumor cells was immunopositive. The presence of any necrosis was evaluated from HE-stained samples.

Statistical analysis

The associations between factors were assessed by Chi-square test (categorical data) (I-IV) and Student's t-test (continuous data) (III, IV). The odds ratio served to examine the strength of the relationships (I). Life-tables were calculated according to the Kaplan-Meier method and compared by the log-rank test. Distant disease-free survival (DDFS) was calculated from date of diagnosis to occurrence of metastases outside the locoregional area or death from breast cancer, whichever came first. Disease-specific survival was calculated from date of diagnosis to death from breast cancer, censoring deaths from intercurrent causes. Overall survival was calculated from date of diagnosis to death from any cause.

Multivariate survival analyses (I, III, IV) were performed with the Cox proportional hazards model. The following covariates were entered: Study I: COX-2 expression (score 0-1 vs. 2-3), age of patient (< 50 vs. ≥ 50 years), number of metastatic lymph nodes (continuous), tumor size in centimeters (continuous), histologic grade (well-differentiated vs. moderately to poorly differentiated), histologic type (nonductal vs. ductal), ER (positive vs. negative), PR status (positive vs. negative), HER-2 amplification (negative vs. positive), Ki-67 expression (< 20% vs. ≥ 20% positive tumor cells) and p53 expression (< 20% vs. ≥ 20% positive tumor cells). Studies II and IV: Age, gender, operation type, and variables related to tumor characteristics such as differentiation grade, tumor stage, and radicality of the

resection were included in this model. Variables with multiple categories were recoded into dichotomous variables by combining categories with comparable prognosis (differentiation grade: well versus moderate to poor; radicality of resection: microscopically radical (R0) versus microscopically nonradical (R1) and macroscopically nonradical (R2); tumor stage: stage I and IIa versus IIb, III, and IV). A P value of 0.05 was adopted as the limit for inclusion of a covariate.

All of the statistical tests were two-sided, and P values of 0.05 or below were considered statistically significant. Statistical analyses were performed with the Statistical Software Package version 9.0 (I, III) or 11.0 (II, IV) (SPSS Inc., Chicago, IL, USA).

RESULTS

COX-2 in breast cancer

Prognostic significance of COX-2 in breast cancer

In Study I, immunoreactivity of COX-2 was evaluated in 1576 invasive breast carcinomas, of which 37.4% stained with high intensity. In Study II, expression of COX-2 protein was analyzed in 231 invasive breast carcinoma specimens, of which 30% stained with high intensity. In both studies, high COX-2 immunoreactivity was localized exclusively to the cytoplasm and sometimes to the perinuclear region of the cancer cells, whereas the stroma was either negative or only weakly positive.

In Study I, elevated expression of COX-2 was significantly more frequent in ductal carcinomas (39.9%) as compared to lobular (29.5%) or special (30.8%; tubular, medullary, mucinous, and papillary) histologic types ($P = 0.0017$). In the group of ductal tumors, COX-2 was associated with high histologic grade ($P < 0.0001$). Elevated COX-2 expression was more common in tumors with large size, negative hormone receptor status, high Ki-67 expression, high p53 expression, or HER-2 amplification ($P < 0.0001$ for each comparison). A significant association also appeared with the presence of axillary lymph node metastases ($P = 0.0001$). No significant association existed between COX -2 and age at diagnosis when 50 years was used as the cut-off value. In Study II, high COX-2 expression was more common in tumors of larger size ($P = 0.041$), negative estrogen receptor status ($P = 0.026$), and negative progesterone receptor status ($P < 0.001$). In the group of ductal tumors, COX-2 expression associated with high histologic grade ($P = 0.003$).

In both studies, elevated COX-2 expression was associated with decreased disease-specific survival. In Study I, among the 1576 breast cancer patients eligible for survival analysis, this was evident when the COX-2 high category was compared to the COX-2 low category ($P < 0.0001$; log-rank test). Kaplan-Meier curves for disease-specific survival are depicted in Figure 4A. In Study II, high

COX-2 expression associated also with decreased disease-specific survival (P = 0.026; log-rank test). Kaplan-Meier curves are depicted in Figure 4B.

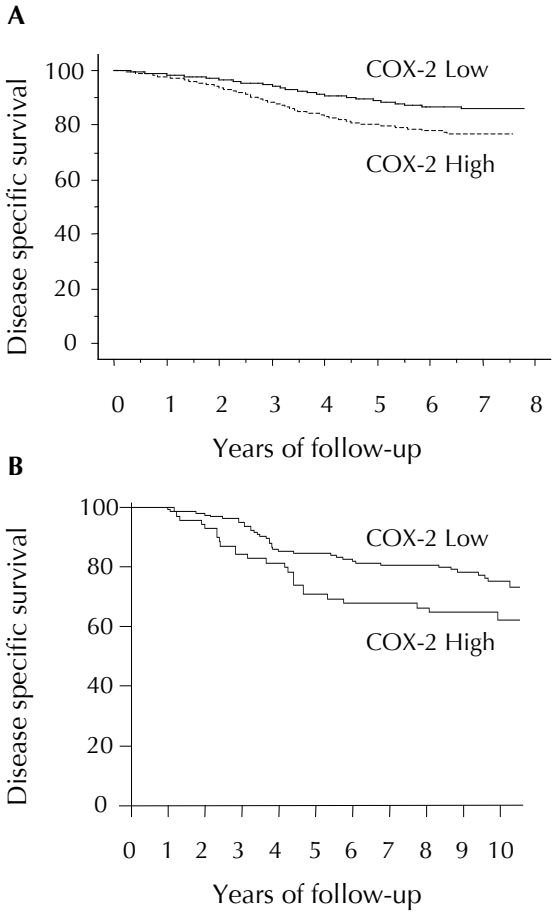

Figure 4: Kaplan-Meier curves for patients with a breast cancer. A: Disease-specific survival of 1576 patients with breast cancer according to COX-2 expression. There were 987 patients with COX-2 low expression and 589 with COX-2 high expression. A statistically significant difference was observed between the groups (P < 0.0001; log-rank test). B: Disease-specific survival of 231 breast cancer patients based on COX-2 expression: 161 patients with COX-2 low expression, 70 with COX-2 high expression. Significant difference was observed between the groups (P = 0.026; log-rank test).

Interestingly, in Study II, we observed that the prognostic impact of COX-2 among subgroups of patients differed. Elevated COX-2 expression predicted poorer survival for patients with ER-positive tumors (P = 0.008; log-rank test) (Figure 5), but not significantly for the hormone receptor-negative ones. A general trend for COX-2 to be a stronger prognostic marker in those subgroups showing features of better prognosis was also evident in other groups (p53 negative/low, no HER-2 amplification, low proliferation rate, and small tumor size). However, dividing

groups by axillary lymph node status made no such difference, since COX-2 expression was associated with both the node-negative and -positive carcinomas (Table 5).

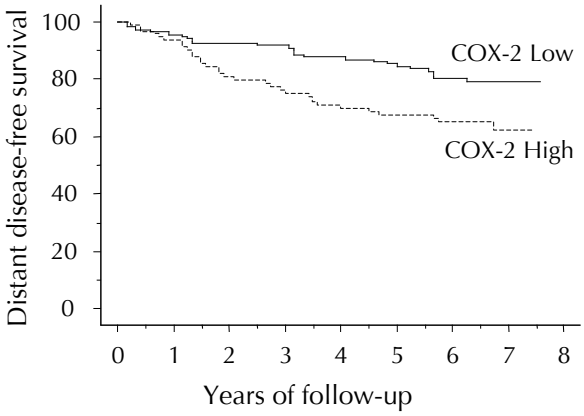

Figure 5: Distant disease-free survival of 232 patients receiving hormonal adjuvant therapy and with estrogen-positive breast cancer, grouped according to COX-2 expression: 137 patients with COX-2 low expression, 95 with COX-2 high expression. Significant difference was observed between the groups (P = 0.008; log-rank test).

Table 5: Five-year DDFS according to COX-2 expression level.

Clinicopathological parameter	**P-value***
Small tumor size (≤ 2 cm)	0.013
Large tumor size (> 2 cm)	Not significant
Node-negative	0.001
Node-positive	0.006
Grade I or 2 (ductal type only)	< 0.05
Grade 3 (ductal type only)	Not significant
Estrogen receptor positive	< 0.0001
Estrogen receptor negative	Not significant
Low rate of proliferation	0.001
High rate of proliferation	Not significant
Negative to low p53 expression	< 0.0001
High p53 expression	Not significant
No amplification of HER-2 oncogene	< 0.0001
Amplification of HER-2 oncogene	Not significant

*Log-rank test or log-rank test for a trend.

Association of COX-2 and MMP-2 protein in breast cancer

Expression of MMP-2 protein was analyzed in 194 invasive breast carcinoma specimens, of which 83.0% stained with high intensity. MMP-2 protein expression localized primarily to the cytoplasm of the cancer cells. High MMP-2 expression associated with reduced disease-specific survival (P = 0.021; log-rank test) as well as with reduced overall survival (P = 0.015; log-rank test). A significant association existed between high COX-2 and high MMP-2 expression (P = 0.003; Chi-square test). It was especially evident that whenever COX-2 expression was high, MMP-2 expression was almost invariably high (56 of 59).

COX-2 in esophageal cancer

Prognostic significance of COX-2 in adenocarcinoma

COX-2 immunoreactivity was detected in 79.3% of the esophageal adenocarcinoma cases (III). COX-2 expression was mainly localized in the neoplastic cells when squamous epithelium of the esophagus was consistently negative or only weakly positive. A significant association appeared between elevated COX-2 expression and development of distant metastases (P = 0.02; Chi-square test) and locoregional recurrences (P = 0.05; Chi-square test). A significant difference in survival was evident between patients in the "COX-2 low" when compared to the "COX-2 high" category (P = 0.002; log-rank test) (Figure 6). As for the role of COX-2 as an independent prognostic factor, univariate Cox regression analysis showed a significant prognostic effect for COX-2 expression, tumor stage, differentiation grade, and radicality of the resection, and multivariate analysis, after adjustment for the other possibly confounding variables, showed no change in the impact of high COX-2 expression on mortality.

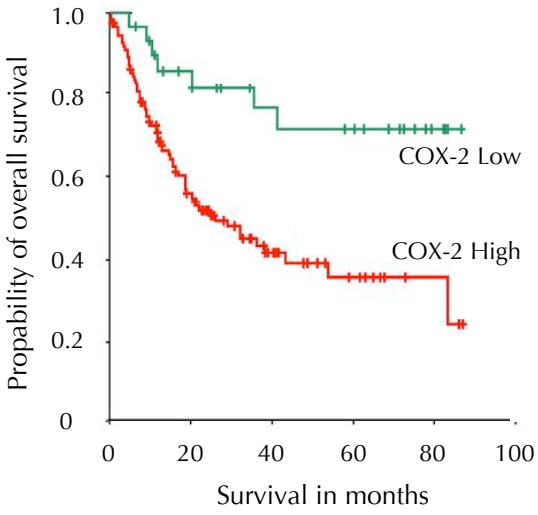

Figure 6: Kaplan-Meier curves for 145 patients with esophageal adenocarcinoma: 30 had low COX-2 expression and 115 high expression. Significant difference was observed between groups (P = 0.002; log-rank test).

Prognostic significance of COX-2 in squamous cell carcinoma

In the 117 esophageal squamous cell carcinoma specimens, expression of COX-2 protein was evaluated by immunohistochemistry. Expression was mainly localized in the neoplastic cells, when nonneoplastic squamous epithelium was consistently negative or only weakly positive. Among the 81 patients receiving no neoadjuvant chemotherapy, COX-2 immunoreactivity was high in 24.7% of the specimens. Among the 36 receiving neoadjuvant treatment, high COX-2 immunoreactivity occurred in 30.6%. In the patient group receiving no neoadjuvant treatment, COX-2 expression was associated with tumor location, with a higher COX-2 expression in distal tumors (P = 0.02; Chi-square test). For the other clinicopathological parameters, no correlation with COX-2 expression was demonstrable, including survival (Figure 7A). In the group of 36 patients receiving neoadjuvant chemotherapy, COX-2 expression was associated with tumor location (P = 0.03; Chi-square test), with male gender (P = 0.02; Chi-square test), and with response to neoadjuvant chemotherapy (P = 0.04; Chi-square test).

Furthermore, a significant association appeared between low COX-2 expression and development of distant metastases (P = 0.03; Chi-square test), with all of the eight distant metastases developing in the low-COX-2 group. Although 13 of the 16 locoregional recurrences developed in that group, the difference was not significant. COX-2 expression did not associate with overall survival in the patient group receiving no neoadjuvant treatment. However, in those 36 patients who did receive it, a significant association appeared between low COX-2 expression and reduced overall survival (P = 0.02; log-rank test) (Figure 7B).

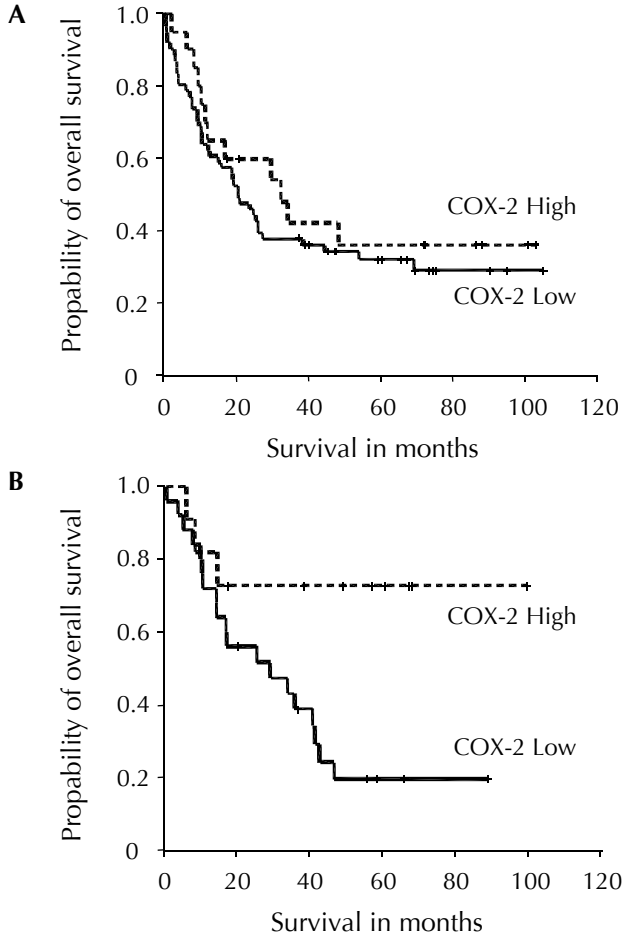

Figure 7: A: Kaplan-Meier curves for 81 patients with squamous cell carcinoma of the esophagus not receiving chemotherapy before surgery: 61 with COX-2 low expression and 20 with COX-2 high expression. No significant difference was observed between groups (P = 0.4, log-rank test). B: Kaplan-Meier curves of 36 patients with squamous cell carcinoma of the esophagus receiving chemotherapy before surgery: 25 with COX-2 low expression and 11 with COX-2 high expression. Significant difference was observed between groups (P = 0.02, log-rank test).

As for the role of COX-2 as an independent prognostic factor in the group of neoadjuvant-treated patients, univariate Cox regression analysis showed a significant prognostic effect for tumor stage, radicality of resection, and low COX-2 expression. In the multivariate analysis, the impact of low COX-2 expression on decreased overall survival persisted after adjustment for the other possibly confounding variables. In patients receiving neoadjuvant treatment, COX-2 expression was re-analyzed and correlated with presence of necrosis and p53 immunopositivity; there appeared, however, no correlation between COX-2 expression and necrosis or p53 immunoreactivity.

DISCUSSION

COX-2 as a prognostic factor in breast carcinoma

Our results in Study I provided the first evidence for COX-2 protein expression in a large series of breast tumors (n = 1576). The database came from a large population-based, multicenter study in Finland using cancer registry data. We detected elevated levels of COX-2 protein expression in 37.4% of the tumors. Similar results were obtained in Study II, where COX-2 protein expression was detected in 30.3% of the samples. In our samples, strong COX-2 expression was localized exclusively in the neoplastic cells, whereas the stroma was either negative or only weakly positive. Consistent with our findings, others have demonstrated immunostaining of COX-2 in epithelial cells of human breast cancer in 17.4% to 63% of the samples (Table 7).

Table 7: COX-2 protein expression in human breast carcinoma.

Frequency of COX-2 expression	
17.4%	Costa *et al.*, 2002
36%	Denkert *et al.*, 2003
40.6%	Wulfing *et al.*, 2003
41.2%	Soslow *et al.*, 2000
43%	Half *et al.*, 2002
48.6%	Spizzo *et al.*, 2003
63%	Boland *et al.*, 2004a

In Study I, COX-2 positivity correlated with many parameters characterizing the aggressive type of breast cancers, i.e., with large tumor size, presence of axillary lymph node metastases, high histological grade, negative hormone-receptor status, high proliferation rate, high p53 expression, and HER-2 amplification. Similarly in Study II, COX-2 expression was associated with tumor size over 2 cm, high histological grade, and negative hormone-receptor status. Several other studies confirm the association between expression of HER-2 and of COX-2 in primary breast cancers (Subbaramaiah *et al.*, 2002; Wulfing *et al.*, 2003; Boland

et al., 2004a), although some studies fail to find such association (Costa *et al.*, 2002; Half *et al.*, 2002; Denkert *et al.*, 2003).

Our main finding showed that in patients with breast cancer elevated level of COX-2 is associated with poor survival. Data in Study II confirm results of Study I. In both studies, COX-2 expression was associated with markers of poor prognosis, such as tumor size over 2 cm, high histological grade, and negative hormone-receptor status. Several recent studies using immunohistochemistry have now confirmed our results. Denkert *et al.* (2003) reported in a study of 221 breast cancer patients that high COX-2 expression is associated with poor survival. Similar to us, they found a significant correlation between COX-2 expression and lymph node status, tumor size, histological grade, and negative ER status, but found no correlation with HER-2. In a study comprising 212 invasive breast cancers, COX-2 overexpression was associated with disease-free and overall survival (Spizzo *et al.*, 2003); COX-2 also correlated with menopausal status and histological tumor type but failed to correlate with any other tumor parameter. In another study, 46 breast carcinomas showed an association of COX-2 expression with lymph node metastasis and angiogenesis but not with any other clinicopathological parameter including HER-2 (Costa *et al.*, 2002), and in addition, high COX-2 expression was associated with short disease-free survival for the subgroup of 26 patients with survival data available. One study reported that in 23 invasive breast cancers, elevated COX-2 expression was associated with diminished overall and disease-free survival (O'Connor *et al.*, 2004). A recent study showed that in invasive breast cancers, and in DCIS, COX-2 expression is associated with high proliferation rate, ER negativity, and with HER-2 positivity (Boland *et al.*, 2004a), but no survival analysis was performed. In contrast to our results, some of the studies found no correlation between COX-2 expression and various clinicopathological parameters including survival (Half *et al.*, 2002; Kelly *et al.*, 2003). For instance, in a study involving 200 human breast carcinomas, expression of COX-2 protein was associated, consistent with our findings, with several markers of aggressive tumor type but not with survival (Wulfing *et al.*, 2003).

Interestingly, the prognostic value of COX-2 expression tended to be significant in only certain subgroups of patients, e.g., in ER-positive, p53-negative, and HER-2-negative tumors, which may indicate that the procarcinogenic effect of COX-2 is not evenly distributed throughout the series. The fact that elevated expression of COX-2 was associated with poor survival in ER-positive tumors is of particular interest. Since COX-2-derived PGE_2 has been implicated in enhancement of stromal cell aromatase expression (Zhao *et al*., 1996), it is possible that elevated COX-2 expression in ER-positive tumors may enhance a growth-promoting microenvironment for the tumor cells by inducing estrogen production via the aromatase pathway in the stromal cells. Consistent with preclinical and clinical observations that COX-2 can induce aromatase expression and estrogen biosynthesis, a recent report showed that regular use of aspirin reduced risk for breast cancer especially in hormone receptor-positive patients (Terry *et al*., 2004). In respect to HER-2, it is interesting to note that COX inhibitors enhance the anti-neoplastic effect of its inhibitors. In a colon carcinoma cell line, the anti-HER-2 monoclonal antibody herceptin and also celecoxib inhibited cell growth *in vitro* and *in vivo*, and the combination of these two drugs was more effective than either agent alone (Mann *et al*., 2001). However, a recent clinical phase II study comprising 12 patients with prior ineffective trastuzumab treatment showed no clinical response from combining celecoxib with trastuzumab (Dang *et al*., 2004). All patients had, however, been heavily pretreated, and their COX-2 expression status was not determined.

With respect to axillary lymph node status, COX-2 predicted significantly poorer survival in both node-negative and node-positive groups. This may reflect the ability of COX-2 to induce metastasis, for example by inducing production and activation of matrix metalloproteinases (Tsujii *et al*., 1997; Takahashi *et al*., 1999). We wished to address this issue in a clinical setting by detecting COX-2 and MMP-2 protein expression by immunohistochemistry in tissue microarrays on consecutively cut sections. This method has certain advantages, since expression of the two enzymes can be detected in a tissue dot diameter of only 0.6 mm. We

showed that expression of COX-2 protein was associated with expression of MMP-2 protein in invasive breast carcinomas. Interestingly, in the group of breast carcinomas with an expression of COX-2 moderate to high, MMP-2 was elevated in 56 of 59 (95%) cases. MMP-2-positive cases were, however, not restricted to COX-2-positive tumors, since MMP-2 was moderate to high in 10 of 133 (77%) of those cases with low COX-2 immunoreactivity. Elevated expression of COX-2 was thus associated almost invariably with elevated expression of MMP-2, but COX-2 must not be the only determinant able to modulate expression of MMP-2 in breast cancer.

Conventional techniques of molecular pathology for analysis of several hundred specimens require much time and higher costs for material. The tissue microarray technique allows a simultaneous analysis of up to 150 tissue biopsies from different tumors on one single tissue chip (Kononen *et al*., 1998). Furthermore, all samples are analyzed simultaneously under the same reaction conditions. Zhang *et al.* (2003) validated the use of tissue microarray technology by comparing ER, PR, and HER-2 immunostaining results for 97 breast tumor specimens using conventional full sections versus tissue microarray sections. Full section versus tissue microarray concordance was 97% for ER, 98% for PR, and 97% for HER-2, making tissue microarray equivalent for analysis of routinely used biomarkers of breast cancer. Furthermore, we have observed in our laboratory that immunostaining of COX-2 in breast cancer specimens is relatively homogenous, and we assume that the tissue microarray technique provides representative results for analysis of immunostaining of COX-2 in breast cancer samples. In immunohistochemistry, however, quantitation is a challenge. To this end, we managed to design a relatively simple scoring method producing results highly concordant between independent evaluators.

In short, our results have provided evidence that COX-2 protein expression is associated in patients with breast cancer with decreased survival. This result came from two separate populations. Interestingly, our data indicate that the prognostic value of COX-2 expression tends to be more marked in certain subgroups of

patients, e.g., in ER-positive tumors compared to those ER-negative. Thus, the tumor-promoting effect of COX-2 may be unevenly distributed throughout a breast cancer population, and that certain subgroups of patients may benefit more from COX-2 targeted therapy.

COX-2 as a prognostic factor in esophageal carcinoma

Our data indicate that in esophageal adenocarcinoma, elevated expression of COX-2 is associated with a more aggressive course of the disease and reduced survival. Furthermore, multivariate analyses suggest that elevated COX-2 expression is an independent prognostic factor for patient survival. In contrast to esophageal adenocarcinoma, in esophageal squamous cell carcinoma COX-2 expression was not associated with survival. These results suggest that the prognostic significance of COX-2 depends on the histological type of esophageal carcinoma. Two recent studies have assessed the connection between COX-2 protein expression and patient outcome in esophageal adenocarcinoma: In concordance with our finding, in one, patients with high COX-2-expressing tumors showed poorer survival (France *et al*., 2004), but the other study found no such association (Lagorce *et al*., 2003). In respect to esophageal squamous cell carcinoma, our results are consistent with those of several other studies finding no association between COX-2 expression and patients outcome (Shamma *et al*., 2000; Kawabe *et al*., 2002; Kuo *et al*., 2003).

We detected high expression of COX-2 protein in 79% of the esophageal adenocarcinomas. Several other studies have reported elevated levels of COX-2 in Barrett metaplasia, dysplasia, and adenocarcinoma (Wilson *et al*., 1998; Shirvani *et al*., 2000; Lagorce *et al*., 2003). Similar to our study on esophageal squamous cell carcinoma, elevated levels of COX-2 mRNA and protein have been reported (Ratnasinghe *et al*., 1999; Zimmermann *et al*., 1999; Shamma *et al*., 2000; Kawabe *et al*., 2002; Kuo *et al*., 2003; Maaser *et al*., 2003). Moreover, a heterogenic intratumoral staining pattern of COX-2 appeared (Maaser *et al*., 2003; Shamma *et al*., 2000), with a significantly higher expression in tumors located in the distal esophagus than in more proximal areas (Kawabe *et al*., 2002). In

esophageal adenocarcinoma, patients with high COX-2-expressing tumors were more likely to develop distant metastases and local recurrences. In contrast, in squamous cell carcinoma of the esophagus, COX-2 expression was associated with none of these clinicopathological variables, in accord with most published data (Shamma *et al*., 2000; Kawabe *et al*., 2002; Kuo *et al*., 2003). However, one study did find an association between low expression of COX-2 and advanced tumor stage and presence of distant metastasis (Kuo *et al*., 2003).

Interestingly, in patients with esophageal squamous cell carcinoma who underwent neoadjuvant chemotherapy, low COX-2 expression was associated with development of distant metastases and reduced overall survival. Our finding contrast with published findings demonstrating a connection between high COX-2 expression and poor survival of patients on such therapy (Ferrandina *et al*., 2002; Kulke *et al*., 2004a). In these studies, however, the biopsy specimens were obtained before neoadjuvant treatment, and in our study the specimens were obtained from surgical resection material after the chemotherapy. Unfortunately, we were unable to include an analysis of the preoperative biopsy specimens in this study. In addition, since the COX-2 staining pattern was relatively heterogeneous in the histological tumor samples, we assume, that staining results of preoperative biopsies might reflect only sampling artefacts. Several *in vitro* studies and one *in vivo* study show that radiation and chemotherapeutic agents can enhance expression of COX-2 (Steinauer *et al*., 2000; Subbaramaiah *et al*., 2000; Subbaramaiah *et al*., 2003; Davis *et al*., 2004). Although there was no statistically significant difference in COX-2 expression between the groups with or without neoadjuvant chemotherapy, we cannot exclude the possibility that COX-2 expression was induced by the chemotherapy. If this was the case, it can be hypothesized that COX-2 signals a favorable response to the neoadjuvant treatment.

Others as well as we have shown a link between COX-2 expression and tumor necrosis (Wolff *et al*., 1998; Ristimäki *et al*., 2001; Bizik *et al*., 2004) and between COX-2 expression and p53 overexpression (Subbaramaiah *et al*., 1999;

Biramijamal *et al.*, 2001; Leung *et al.*, 2001; Kawabe *et al.*, 2002; Gallo *et al.*, 2003; Erkinheimo *et al.*, 2004). To address this issue, we evaluated any possible correlation between COX-2 expression and necrotic area or p53 overexpression in tumors derived from chemotherapy-treated esophageal squamous cell carcinoma patients. We found, however, no association between COX-2 expression and tumor necrosis or p53 immunoreactivity. It thus seems that COX-2 expression is independent of the necrotic process and of loss of p53 function in this series.

In conclusion, our study was the first to report high expression of COX-2 to be associated with reduced survival in adenocarcinoma of the esophagus. In contrast, in esophageal squamous cell carcinoma, COX-2 expression was not associated with patient outcome. However, low COX-2 expression was associated with poor prognosis in patients with esophageal squamous cell carcinoma who received neoadjuvant chemotherapy. In summary, our results suggest that the prognostic significance of COX-2 depends on histological type of esophageal carcinoma and on treatment applied.

CONCLUDING REMARKS

Our study shows that overexpression of COX-2 seems to contribute to tumorigenesis in the breast. Although no conclusions can be drawn from the association between COX-2 expression and poor outcome in regard to treatment, the present findings support efforts to initiate clinical trials on the efficacy of COX-2 inhibitors in adjuvant treatment of breast cancer. Our results also suggest that COX-2 inhibitors may be more effective in treatment of certain subgroups of patients. Since COX-2 is overexpressed in HER-2-positive breast cancers, selective COX-2 inhibitors could be evaluated in this patient group. In addition, our results provide a basis for study of the predictive value of COX-2 expression in clinical trials assessing the efficacy of novel aromatase inhibitors versus the classical anti-estrogen tamoxifen.

Our results show that overexpression of COX-2 also seems to contribute to tumorigenesis in esophageal adenocarcinoma, but in esophageal squamous cell carcinoma such a connection is less clear. Unexpectedly, low COX-2 expression was associated with poor prognosis in patients with esophageal squamous cell carcinomas who received neoadjuvant chemotherapy, indicating that in esophageal cancer the prognostic role of COX-2 seems to depend on histological type and on treatment applied. Differences can be explained by the fact that each malignancy may have its own pathway of carcinogenesis and its own set of risk factors and protective exposures. Since esophageal cancers are highly lethal, current treatment protocols are insufficient, and new treatment modalities are needed. Research on COX-2 in esophageal cancers offers a new approach to these patients. However, based on our results, selective COX-2 inhibitors seem more promising for esophageal adenocarcinoma than for esophageal squamous cell carcinoma.

In patients with esophageal squamous cell carcinomas who received neoadjuvant chemotherapy, low COX-2 expression was associated with poor prognosis. Association of COX-2 with tumor chemoresistance has two potentially important consequences: First, if this association is evident in pre-treatment biopsy samples,

it may serve as a marker predicting chemotherapy treatment failure. Second, use of COX-2 inhibitors may enhance the effects of therapy. Our study indicates that even if a combination of COX-2 inhibitors with radio/chemotherapy seems an interesting approach, this may not be the case for all cancer types. Whether COX-2 can predict response to chemo- or radiotherapy requires further investigation.

In summary, COX-2 may serve as a biomarker for certain neoplastic conditions. Alternatively, selective COX-2 inhibitors may prove useful in chemoprevention or in treatment of breast or esophageal cancers, although recent results showing increased cardiovascular toxicities among users of COX-2 inhibitors make this approach less promising. Possible cardiovascular effects will need to be taken into account in assessment of these drugs' potential to prevent or treat neoplasias.

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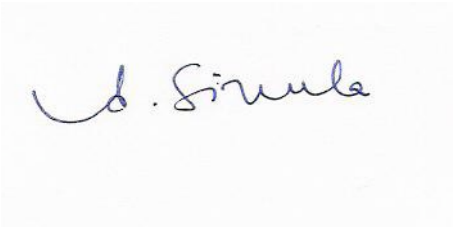
Maikari, March 20th 2005



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