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Advances in Brief

Cyclooxygenase-2 Expression Is Related to Nuclear Grade in Ductal Carcinoma *in Situ* and Is Increased in Its Normal Adjacent Epithelium[1]

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Abstract

Cyclooxygenase-2 (COX-2) is emerging as an important cancer biomarker and is now an experimental target for solid tumor treatment. However, no study has exclusively focused on COX-2 expression in early lesions such as ductal carcinoma *in situ* (DCIS). We examined COX-2 expression by immunohistochemistry in 46 cases of women undergoing surgical resection for DCIS. We found that COX-2 expression was detected in 85% of all DCIS specimens, with increased COX-2 staining correlating with higher nuclear grade. Strikingly, COX-2 staining intensity in the normal adjacent epithelium was stronger than in the DCIS lesion itself. Our observations demonstrate that COX-2 is up-regulated in the normal adjacent epithelium and supports the hypothesis that the surrounding epithelial tissue is part of the disease process in DCIS.

Introduction

Many studies demonstrate that COX-2[4] expression is up-regulated in numerous invasive cancers such as bronchial, colon, and breast (1). The up-regulation of COX-2 expression is induced in many tissues by various growth factors, cytokines, and tumor promoters (2–5). COX-2 metabolizes the cell membrane fatty acid, arachadonic acid, to yield diffusible PGs. PGE_2 has been found to be the most abundant PG produced by epithelial cells and is released through the basolateral cell compartment into the underlying stroma (6). *In vitro* studies have demonstrated that PGE_2 can stimulate epithelial cell proliferation and motility, thereby contributing to neoplastic progression (7). In breast cancer, data on the clinical implication of COX-2 expression is limited. Thus far, studies have shown that COX-2 is expressed in invasive breast cancer and correlates with high nuclear grade and HER-2/neu overexpression (2, 8–10). Because nuclear grade and HER-2/neu overexpression in invasive tumors are associated with recurrence and survival, COX-2 expression may also be associated with tumors with adverse outcome. COX-2 overexpression is also explored as a potential therapeutic target. There is a paucity of data on the relationship between COX-2 expression and preinvasive breast cancer lesions such as DCIS. In a study of invasive cancer and a small number of DCIS lesions, Half *et al.* (9) have recently demonstrated that DCIS adjacent to invasive cancer expressed higher COX-2 levels than its invasive component, suggesting that COX-2 expression may be an early event in breast carcinogenesis. The pattern of COX-2 expression in DCIS alone, however, is not well characterized. With the advent of screening mammography, >50% of new diagnoses are preinvasive cancer or DCIS. Numerous studies suggest that surgical margins and tumor nuclear grade in DCIS play an important role in recurrence during the first 5–10 years of follow-up (11, 12). A number of other markers, including ER, PgR, p53, and HER2/neu, have been examined, none of which have been shown to predict the risk of breast cancer recurrence in DCIS (12–14). ER, however, has recently been shown to be a good predictive marker of Tamoxifen benefit in the DCIS setting. Given the differential expression of COX-2 in invasive breast cancer and the interest in clinical breast cancer applications for COX-2 inhibitors, we evaluated the prevalence of COX-2 expression in DCIS. Given that PGs are diffusible factors that induce both autocrine and paracrine responses, we also evaluated COX-2 expression in the surrounding normal breast epithelium.

Materials and Methods

Tissue Samples. We analyzed a series of primary human DCIS slides ($n = 46$) obtained from the surgical pathology laboratory of the University of California, San Francisco, and California Pacific Medical Center. The samples represented an even distribution of DCIS nuclear grade: low ($n = 14$); intermediate ($n = 18$); and high ($n = 14$).

Tissue Preparation and Immunohistochemistry. Five-μm sections were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on SuperFrost-Plus microscope slides (Fisherbrand, Fisher Scientific, Pittsburgh, PA). Specimens were stepwise deparaffinized in xylene and rehydrated in descending alcohols. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in methanol for 10 min. Sections were then microwaved for antigen retrieval for 10 min in a 10 mM citrate buffer (pH 6.0). Cases were stained for COX-2, ER, PgR, and HER/neu overexpression. For COX-2, sections were incubated overnight at 4°C with mouse antihuman monoclonal antibodies (No.160112; Cayman Chemical, Ann Arbor, MI) diluted to 1:200 in 1% BSA in PBS. Control sections were incubated with a human COX-2 control peptide (40ug/ml; Cayman Chemical). Sections were rinsed in 0.05% Tween 20 in PBS followed by incubation with biotinylated horse antimouse IgG antibodies (No. BA-2000; Vector Laboratories, Burlingame, CA) and diluted to 1:200 in 1% BSA in PBS. Slides were then rinsed in PBS and incubated in avidin-biotin horseradish peroxidase complex (No. PK-6100, Vectastain Elite ABC kit; Vector Laboratories) at a 1:100 dilution in 1% BSA in PBS for 30 min. Specimens were rinsed in 0.05% Tween 20 in PBS then incubated with 3,3′-diaminobenzidine chromogenic substrate (No. D-5905; Sigma Chemical, St. Louis, MO) for 4 min. Sections were counterstained in hematoxylin, stepwise dehydrated through graded alcohols, and cleared in xylene before mounting using Permount. Sections from prostate tumor were stained with each experiment as a control. For ERs, monoclonal mouse antihuman ER antibodies (clone 1D5; Dako Corporation, Carpinteria, CA) were applied at 1:400 overnight at 4°C after microwave antigen retrieval. Detection was performed as above. A known ER-positive breast cancer was run as a control. For PgRs, monoclonal antihuman PgR receptor antibodies (clone 1A6; Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) was used at 1:25 overnight at 4°C. Detection was performed as above. Cytospins of T47D cell lines were used as positive controls. For HER2/neu

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[4] The abbreviations used are: COX-2, cyclooxygenase-2; PG, prostaglandin; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; PgR, progesterone receptor; QI, quantity and intensity.

Table 1 *Clinicopathological variables in DCIS*

	Low grade (*n*)	Intermediate grade (*n*)	High grade (*n*)
Mean age (yr)	65	57	60
DCIS (*n*)	14	18	14
Tumor size (mm)	6	15	11.5
Necrosis	0	8	13
ER			
Negative	0	0	6
Positive	13	14	6
ND[a]	1	4	2
PgR			
Negative	2	4	6
Positive	12	12	8
ND	0	2	0
Her2/neu			
Negative	8	16	7
Positive	1	1	6
ND	5	1	0
COX-2			
Intensity 0	5	1	1
Intensity 1	2	3	2
Intensity 2	5	11	7
Intensity 3	2	3	4

[a] ND, not determined.

overexpression, sections were pretreated with Ficin (Zymed Laboratories, South San Francisco, CA), followed by 4°C overnight incubation at 1:200 dilution of monoclonal anti-c-erbB-2 antibodies (clone TAB250; Zymed Laboratories). Detection was performed as above. Cytospins of BT474 and MCF7 cell lines were used as positive and negative controls. Because only one case presented with a 2+ Her2/neu score, fluorescent *in situ* hybridization was not performed.

Evaluation of COX-2 Immunostaining. Estimation of COX-2 expression was performed as previously described, based on the German ImmunoReactive Score (1, 15). The COX-2 staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong) and COX-2 staining quantity (0, no staining; 1–1 to 10%; 2–11 to 50%; 3–51 to 80%; and 4–81 to 100%) were evaluated by light microscopy without any knowledge of the patients' clinical data. The overall score was expressed as the summation of the intensity and quantity scores and defined as the QI product with a range of 0–12. When the intensity of multifocal patches was significantly different between foci, the average of the least intense and most intense staining was recorded.

Statistical Methods. Contingency table analysis based on χ^2 statistics was used to test the associations between COX-2 stain intensity, QI product (intensity × quality) *versus* nuclear grade and age. Furthermore, correlations between COX-2 and ER, PgR, and/or HER 2/neu status (positive or negative) were evaluated. Average values for intensity, quantity, and their product were calculated for each specimen type (tumor and adjacent epithelium) and compared pair-wise (*i.e.*, tumor *versus* adjacent) using a paired *t* test.

Results

Patient Characteristics and Clinical/Pathological Variables. Table 1 shows the clinical and pathological variables of the cases studied. Median age of the patients in this group was 60 years old (range, 25–78 years). One-third of patients were <50 years old and two-thirds > 50 years old, similar to national demographics of DCIS. The 46 cases of DCIS evaluated were evenly distributed by nuclear grade. The size of DCIS lesions ranged from 1 to 49 mm, with the average size doubling from low-grade to intermediate and high-grade lesions. Necrosis was not observed in low nuclear grade DCIS lesions (0 of 14) but prevalent in high nuclear grade lesions (13 of 14).

COX-2 Expression in DCIS. COX-2 expression was observed in 85% of DCIS lesions with increased staining intensity associated with higher grade lesions ($P = 0.048$). Fig. 1*A* demonstrates the difference in COX-2 intensity between low and higher grade lesions, including both intermediate and high nuclear grade DCIS. Representative examples of COX-2 expression are shown in Fig. 1*B*. Although COX-2 expression within an individual lesion was fairly uniform, within each grade, there was a range of staining intensity from 0 to 3+. The QI product (which ranged from 0 to 12) also increased with nuclear grade but did not reach statistical significance ($P = 0.26$). No other clinicopathological characteristics, including patient's age, ER, PgR,

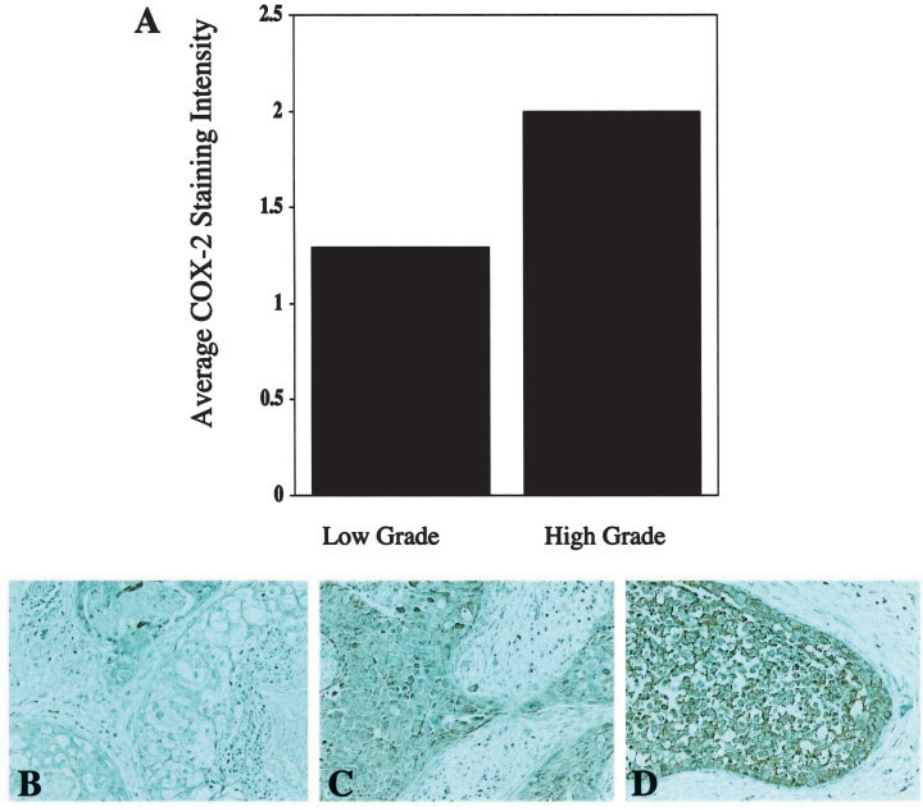

Fig. 1. COX-2 staining intensity increased with DCIS nuclear grade (*A*). Representative examples of low (*B*), intermediate (*C*), and high (*D*) COX-2 immunohistochemistry staining intensity of high nuclear grade DCIS.

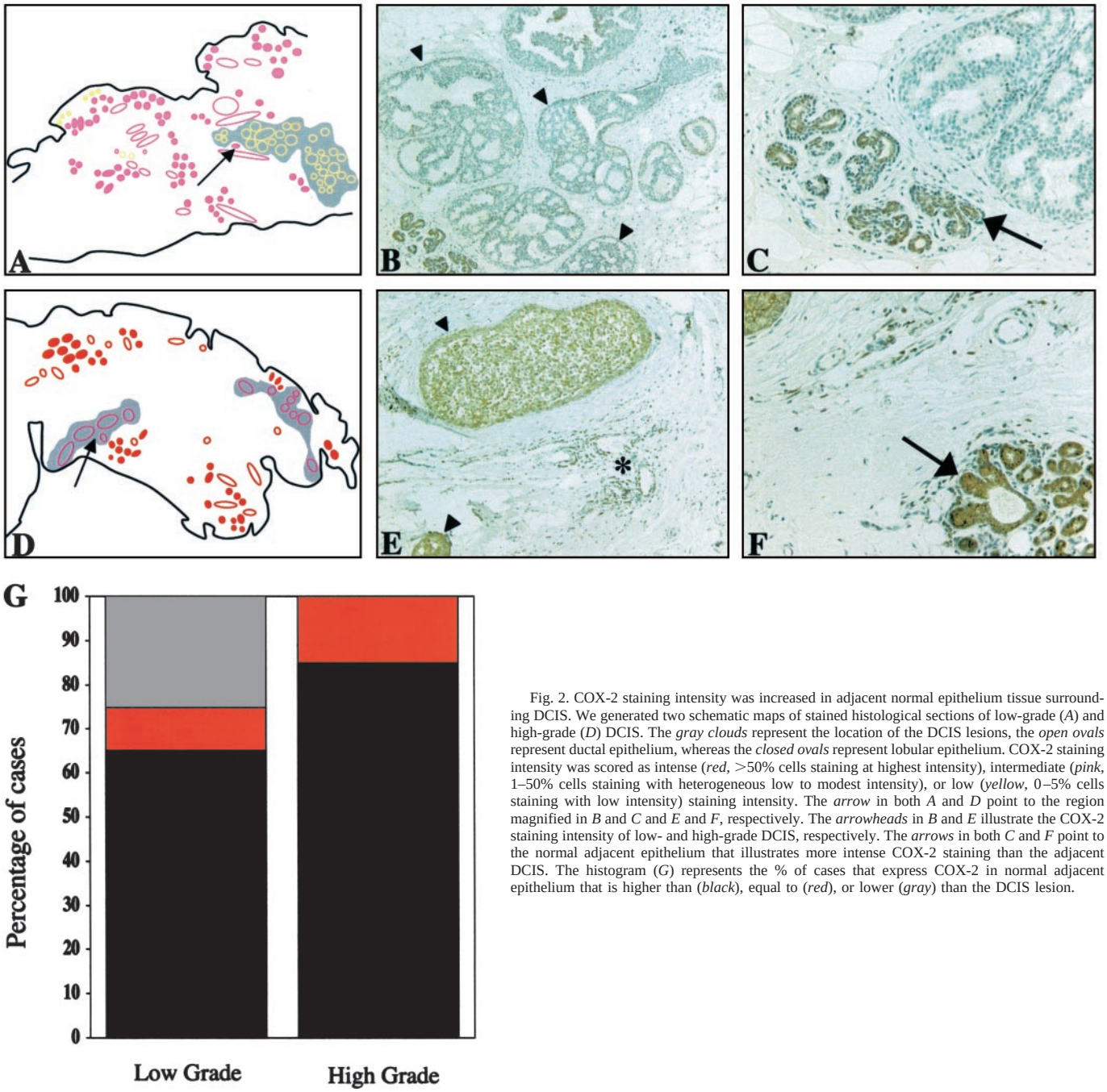

Fig. 2. COX-2 staining intensity was increased in adjacent normal epithelium tissue surrounding DCIS. We generated two schematic maps of stained histological sections of low-grade (*A*) and high-grade (*D*) DCIS. The *gray clouds* represent the location of the DCIS lesions, the *open ovals* represent ductal epithelium, whereas the *closed ovals* represent lobular epithelium. COX-2 staining intensity was scored as intense (*red*, >50% cells staining at highest intensity), intermediate (*pink*, 1–50% cells staining with heterogeneous low to modest intensity), or low (*yellow*, 0–5% cells staining with low intensity) staining intensity. The *arrow* in both *A* and *D* point to the region magnified in *B* and *C* and *E* and *F*, respectively. The *arrowheads* in *B* and *E* illustrate the COX-2 staining intensity of low- and high-grade DCIS, respectively. The *arrows* in both *C* and *F* point to the normal adjacent epithelium that illustrates more intense COX-2 staining than the adjacent DCIS. The histogram (*G*) represents the % of cases that express COX-2 in normal adjacent epithelium that is higher than (*black*), equal to (*red*), or lower (*gray*) than the DCIS lesion.

HER2/neu, or necrosis, demonstrated a correlation with COX-2 expression.

COX-2 Expression in Normal Adjacent Epithelium. To illustrate the geographic relationship between COX-2 expression in DCIS lesions and in normal adjacent epithelia, we generated schematic maps of the stained histological sections (Fig. 2, *A* and *D*). These two schematic maps represent a low power view of breast tissue from patients with low grade DCIS (Fig. 2*A*) and high grade DCIS (Fig. 2*D*). The schematic map integrates three types of information: the type of tissue (ductal or lobular); the location of DCIS; and the intensity of COX-2 staining. The closed and open structures represent the type of epithelial elements (lobular and ductal structures, respectively), the gray cloud represents the area of DCIS, and the color of the epithelial elements is coded to reflect COX-2 staining intensity (yellow, pink, and red for low, intermediate, and intense staining, respectively). Note that there was a paucity of COX-2 staining in the low grade DCIS lesion (arrow head), although there was evident COX-2 staining in the adjacent normal epithelium (arrow; Fig. 2, *A–C*). This pattern of increased staining intensity in the normal adjacent epithelium was even more striking in high-grade DCIS (Fig. 2, *D–F*). Representative immunohistological staining of these patterns are illustrated for low grade DCIS (Fig. 2, *B* and *C*) and high-grade DCIS (Fig. 2, *E* and *F*). When sufficient tissue was present, we examined COX-2 expression in epithelium > 1 cm distal to the original lesion. In high-grade lesions, we observed that the increase in COX-2 expression often extended beyond 1 cm from the lesion but diminished with distance (data not shown). We noted that although there was no detectable COX-2 staining of stromal fibroblasts when

immune cells were found associated with higher grade DCIS lesions, they often expressed COX-2 (Fig. 2*E*, asterisk). In the population of DCIS lesions we examined, the distinctive pattern of increased expression of COX-2 in normal adjacent epithelium relative to the DCIS lesion was prevalent. The pattern was particularly conspicuous in high-grade lesions, as a group, as demonstrated by Fig. 2*G*. Note that in all higher grade lesions (including intermediate and high nuclear grade DCIS), the level of COX-2 expression in the normal adjacent epithelium was never less than that seen in the DCIS lesion but was always at least equal (15%) or greater (85%).

Discussion

We report the largest DCIS series of COX-2 expression. This study demonstrates two important aspects of COX-2 expression in premalignant lesions. The first observation is that COX-2 expression in DCIS is common and expression increases with nuclear grade. The second and possibly more significant is the finding that the COX-2 intensity in the normal adjacent epithelium is stronger than in the lesion itself and correlates with DCIS nuclear grade.

Recurrence of DCIS after surgical resection usually occurs in the same breast quadrant and has been largely shown to be a clonal derivative from the primary lesion (16), and surgical margin width $>$ 10 mm is associated with a lower recurrence rate (11, 17, 18). However, accurate assessment of the three-dimensional tumor involvement is technically difficult, and therefore, tumor characteristics as well as reactive stromal indices may provide additional information to the extent of malignant participation. Our observations that COX-2 is up-regulated in the surrounding epithelial tissue raise the strong possibility that the adjacent normal epithelium is part of the disease process in DCIS. It is unlikely that COX-2 alone is responsible for promoting malignant growth, however, COX-2 might play a role mediating a field effect. In colorectal cancer, autocrine and paracrine PG signaling in the tumor microenvironment have been well studied and supports a role for COX-2 inhibitors in cancer treatment and prevention, as discussed in a recent review by Gupta and DuBois (19). The role of the microenvironment in inducing and/or maintaining COX-2 expression in preinvasive lesions of the breast such as DCIS has not been well studied, but our results suggest that it is indeed possible that a similar process may be operative in breast cancer initiation and warrants additional investigation. If a field effect is indeed present, this could explain why recurrences are reduced in DCIS when wide margins are obtained at the time of surgical resection.

Because COX-2 expression was prevalent it was difficult to conclude any statistically significant relationship with other variables such as age, tumor size, ER, PgR, or HER2/neu. Although *in vitro* studies have shown COX-2 expression associated with HER-2/neu overexpression, our *in vivo* DCIS study does not support the association with COX-2 expression and HER-2/neu (2). This is likely attributable to prevalence of COX-2 expression seen in the intermediate nuclear grade group, which did not show elevated HER2/neu overexpression. In addition, we only found 50% of high-grade lesions to be HER-2/neu positive, which is lower than has been reported previously (20). Because COX-2 expression was much more prevalent than HER 2/neu in our study, it is likely that other factors are responsible for COX-2 expression observed here.

This study, which focuses exclusively on DCIS and COX-2 expression, provides some interesting insights and guidance for future research and clinical applications. We demonstrate first that COX-2 expression correlates with DCIS nuclear grade. Second, and most interestingly, COX-2 was overexpressed in histologically normal epithelium surrounding DCIS lesions. These data suggest that in the setting of large clinical DCIS trials, COX-2 expression in adjacent tissue is worth exploring as a marker for recurrence and, potentially, as a therapeutic target.

References

1. Soslow, R. A., Dannenberg, A. J., Rush, D., Woerner, B. M., Khan, K. N., Masferrer, J., and Koki, A. T. COX-2 is expressed in human pulmonary, colonic, and mammary tumors. Cancer (Phila.), *89:* 2637–2645, 2000.
2. Subbaramaiah, K., Norton, L., Gerald, W., and Dannenberg, A. J. Cyclooxygenase-2 is overexpressed in HER-2/neu-positive breast cancer: evidence for involvement of AP-1 and PEA3. J. Biol. Chem., *277:* 18649–18657, 2002.
3. Matsurra, H., Sakaue, M., Subbaramaiah, K., Kamitani, H., Eling, T. E., Dannenberg, A. J., Tanabe, T., Inoue, H., Arata, J., and Jetten, A. M. Regulation of cyclo-oxygenase-2 by interferon γ and transforming growth factor α in normal human epidermal keratinocytes and squamous carcinoma cells. Role of mitogen-activated protein kinases. J. Biol. Chem., *274:* 29138–29148, 1999.
4. Huang, J. C., Liu, D. Y., Yadollahi, S., Wu, K. K., and Dawood, M. Y. Interleukin-1 β induces cyclo-oxygenase-2 gene expression in cultured endometrial stromal cells. J. Clin. Endocrinol. Metab., *83:* 538–541, 1998.
5. Crofford, L. J., Tan, B., McCarthy, C. J., and Hla, T. Involvement of nuclear factor κB in the regulation of cyclo-oxygenase-2 expression by interleukin-1 in rheumatoid synovocytes. Arthritis Rheum., *40:* 226–236, 1997.
6. Coffey, R. J., Hawkey, C. J., Damstrup, L., Graves-Deal, R., Daniel, V. C., Dempsey, P. J., Chinery, R., Kirkland, S. C., DuBois, R. N., Jetton, T., and Morrow, J. D. Epidermal growth factor receptor activation induces nuclear targeting of cyclooxygenase-2, basolateral release of prostaglandins, and mitogenesis in polarizing colon cancer cells. Proc. Natl. Acad. Sci. USA, *94:* 657–662, 1997.
7. Sheng, H., Shao, J., Washington, M. K., and DuBois, N. Prostaglandin E2 increases growth and motility of colorectal carcinoma cells. J. Biol. Chem., *276:* 18075–18081, 2001.
8. Ristimaki, A., Sivula, A., Lundin, J., Lundin, M., Salminen, T., Haglund, C., Joensuu, H., and Isola, J. Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. Cancer Res., *62:* 632–635, 2002.
9. Half, E., Tang, X. M., Gwyn, K., Sahin, A., Wathen, K., and Sinicrope, F. A. Cyclooxygenase-2 expression in human breast cancers and adjacent ductal carcinoma *in situ.* Cancer Res., *62:* 1676–1681, 2002.
10. Parrett, M. L., Harris, R. E., Joarder, F. S., Ross, M. S., Clausen, K. P., and Robertson, F. M. Cyclooxygenase-2 expression in mans breast cancer. Int. J. Oncol., *10:* 503–507, 1997.
11. Silverstein, M. J., Lagios, M. D., Groshen, S., Waisman, J. R., Lewinsky, B. S., Martino, S., Gamagami, P., and Colburn, W. J. The influence of margin width on local control of ductal carcinoma *in situ* of the breast. N. Engl. J Med., *340:* 1455–1461, 1999.
12. Silverstein, M. J., Lagios, M. D., Martino, S., Lewinsky, B. S., Craig, P. H., Beron, P. J., Gamagami, P., and Waisman, J. R. Outcome after invasive local recurrence in patients with ductal carcinoma *in situ* of the breast. J. Clin. Oncol., *16:* 1367–1373, 1998.
13. Silverstein, M. J. Ductal Carcinoma *in situ* of the breast: controversial issues. Oncologist, *3:* 94–103, 1998.
14. Silverstein, M. J., and Masetti, R. Hypothesis and practice: are there several types of treatment for ductal carcinoma *in situ* of the breast? Recent Results Cancer Res., *152:* 105–122, 1998.
15. Krajewska, M., Krajewski., S., Epstein., J. I., Shabaik, A., Sauvageot, J., Kitada, S., and Reed, J. C. Immunohistochemical analysis of bcl-2, bcl-X, and mcl-1 expression in prostate cancers. Am. J. Pathol., *148:* 1567–1576, 1996.
16. Waldman, F. M., DeVries, S., Chew, K. L., Moore, D. H., Kerlikowske, K., and Ljung, B. M. Chromosomal alterations in ductal carcinomas *in situ* and their *in situ* recurrences. J. Nat. Cancer Inst. (Bethesda), *92:* 313–320, 2000.
17. Lagios, M. D. Heterogeneity of duct carcinoma *in situ* (DCIS): relationship of grade and subtype analysis to local recurrence and risk of invasive transformation. Cancer Lett., *90:* 97–102, 1995.
18. Silverstein, M. J. Ductal carcinoma *in situ* of the breast. Annu. Rev. Med., *51:* 17–32, 2000.
19. Gupta, R. A., and DuBois, R. N. Colorectal cancer prevention and treatment by inhibition of cyclo-oxygenase-2. Nat. Rev. Cancer, *1:* 11–21, 2001.
20. Schimmelpenning, H., Eriksson, E., Falkmer, U. G., Azavedo, E., Svane, G., and Auer, G. U. Expression of the c-erb B-2 proto-oncogene product and nuclear DNA content in benign and malignant breast parenchyma. Virchows Arch. A Pathol. Anat. Histol., *420:* 433–440, 1992.