

ORIGINAL ARTICLE

Cyclo-oxygenase 2 expression is associated with angiogenesis and lymph node metastasis in human breast cancer

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Aims: Cyclo-oxygenases 1 and 2 (COX-1 and COX-2) are key enzymes in prostaglandin biosynthesis. COX-2 is induced by a wide variety of stimuli, and present during inflammation. COX-2 overexpression has been observed in colon, head and neck, lung, prostate, stomach, and breast cancer. In colon and gastric cancer, COX-2 expression was associated with angiogenesis. The aim of this study was to determine the relation between COX-2 expression and angiogenesis in breast cancer, and to correlate the expression of this enzyme with classic clinicopathological parameters.

Methods: COX-2 expression was investigated by immunohistochemistry and western blotting analysis. The expression of COX-2 was then related to age, histological grade, nodal status, oestrogen receptor status, p53 expression, c-erb-B2 overexpression, mitotic counts, MIB-1 labelling index, apoptotic index, sialyl-Tn expression, transforming growth factor α expression, microvessel density, and disease free survival in 46 patients with invasive ductal breast carcinoma.

Results: By means of immunohistochemistry, COX-2 expression was detected in eight of the 46 carcinomas studied. Western blotting showed COX-2 protein expression in the same breast tumours, but not in normal adjacent tissues. The density of microvessels immunostained with anti-F-VIII related antigen was significantly higher in patients with COX-2 expression than in those without expression ($p = 0.03$). In addition, COX-2 was significantly associated with the presence of sialyl-Tn expression ($p = 0.02$), lymph node metastasis ($p = 0.03$), a high apoptotic index ($p = 0.03$), and a short disease free survival ($p = 0.03$) in univariate analyses.

Conclusions: These data suggest that COX-2 expression is associated with angiogenesis, lymph node metastasis, and apoptosis in human breast cancer. Moreover, these results warrant further studies with larger series of patients to confirm the association with short disease free survival in patients with breast cancer.

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Arachidonic acid metabolites, such as prostaglandins, thromboxanes, and various lipoxigenase products, are produced in many tissues and are responsible for a wide variety of biological responses. They are potent mediators, affecting several signal transduction pathways that modulate cellular adhesion, growth, and differentiation.^{1, 2}

Cyclo-oxygenase (COX) (otherwise known as prostaglandin endoperoxide synthase) catalyses the conversion of arachidonic acid to prostaglandin endoperoxide (prostaglandin H₂). This is the rate limiting step in prostaglandin and thromboxane biosynthesis. Two isoforms of prostaglandin synthase have been identified and are often referred to as COX-1 and COX-2.³ COX-1 is constitutively expressed in most tissues and is thought to be involved in maintaining cellular homeostasis.⁴ In contrast, COX-2 is frequently undetectable at baseline in normal tissues, but can undergo rapid induction in response to a variety of stimuli, including mitogens, hormones, cytokines, and growth factors. The effects of both cyclo-oxygenases are mediated via tyrosine kinase, protein kinase C, and/or protein kinase A signal transduction pathways.⁵

Overexpression of COX-2 and high concentrations of prostaglandins have been associated with chronic inflammatory diseases, such as rheumatoid arthritis,^{6, 7} and several types of human cancer, including colon, head and neck, lung, bladder, prostate, stomach, and breast cancer.^{8–18} During cancer progression, prostaglandins can mediate several mechanisms, including cell proliferation,¹⁹ apoptosis,^{20, 21} modulation of the immune system,^{22, 23} and angiogenesis.^{24, 25} Angiogenesis or neovascularisation, is the formation of new blood vessels from pre-existing ones.^{26, 27} In physiological processes, angiogenesis

plays an important role in the female reproductive cycle (ovulation, menstruation) and in tissue growth and repair. In chronic inflammatory diseases, the formation of new blood vessels maintain the inflammatory state, by transporting inflammatory cells and supplying oxygen and nutrients to the proliferative inflamed tissue.²⁸

“Increased concentrations of prostaglandins E₂, a major product of cyclo-oxygenase 2, have been reported in human breast cancer and in experimental murine mammary tumour models”

During tumorigenesis, angiogenesis is important for tumour growth and metastasis. Any increase in a tumour mass must be preceded by an increase in the vascular supply, to deliver nutrients and oxygen to the tumour. Angiogenesis is mediated by a change in the local equilibrium between positive and negative angiogenic factors. Among the most important proangiogenic factors are vascular endothelial growth factor (VEGF), fibroblast growth factor, platelet derived growth factor, and angiopoietin 1.^{26, 27}

Although the mechanisms underlying the contribution of COX-2 to tumorigenesis are still unclear, an association

Abbreviations: COX, cyclo-oxygenase; ER, oestrogen receptor; F-VIII, factor VIII related antigen; HPF, high power field; STn, sialyl Tn; TGF- α , transforming growth factor α ; VEGF, vascular endothelial growth factor

between COX-2 overexpression and angiogenesis has been demonstrated in two types of cancer—colorectal and gastric cancer.^{23, 24} Overexpression of COX-2 in a colorectal cancer cell line was shown to stimulate the production of angiogenic factors, such as VEGF.²⁴ In a recent study, COX-2 expression was associated with increased angiogenesis in gastric cancer.²⁵

Increased concentrations of prostaglandins E₂, a major product of COX-2, have been reported in human breast cancer and in experimental murine mammary tumour models.^{29, 30} Several studies with murine mammary tumour cells indicate that prostaglandins E₂ may have a multifunctional role in controlling growth, metastasis, and the host immune response in breast cancer.^{29–31} The COX-2 expression profile has also been evaluated in human breast cancer samples,^{32, 33} but the causal role and molecular mechanisms of the enzyme during breast tumorigenesis are still not well defined.

Therefore, the aim of our present study was to investigate whether there is an association between COX-2 expression and angiogenesis, in addition to other clinicopathological parameters of aggressiveness in human breast cancer.

MATERIAL AND METHODS

Case selection

Our study was carried out in 46 women, aged 28–77 years (mean, 56; SE, 1.3), with primary breast carcinomas diagnosed in our institute. Surgical specimens obtained by mastectomy were 10% buffered formalin fixed and paraffin wax embedded; 4 µm sections were cut and stained with haematoxylin and eosin. The histological grading was performed using the modified criteria of Bloom and Richardson, as described by Elston and Ellis.³⁴ The disease free survival was defined as the period between the diagnosis and death of the patient. Only patients with at least a 24 month follow up period were included in the survival analysis. Frozen tumour material was obtained from 19 of the 46 patients, in addition to two matched normal breast tissue samples, and these samples were used for western blotting.

Mitotic counts and the evaluation of apoptosis

Mitotic counts and the evaluation of the apoptotic index were performed according to the method described and validated by Liu *et al.*³⁵ Briefly, to assess the mitotic counts we only considered those mitotic cells in which (1) the nuclear membrane was absent, (2) the condensed chromosomes showed hair-like extensions of nuclear material, (3) there was no central clear zone, and (4) there was a basophilic staining of the cytoplasm. To evaluate the apoptotic index, we considered only those apoptotic cells that showed the following morphological criteria: cell shrinkage, chromatin condensation, nuclear fragmentation, and lack of inflammatory cells.

To obtain the mitotic counts, we counted the number of mitotic figures in 10 high power fields (HPFs) using a ×40 objective and ×10 ocular, numerical aperture 0.74 mm² in the hot spot areas of the tumours, which were defined as the areas with the highest cellularity and number of mitotic figures.

To calculate the apoptotic index, in each tumour we assessed only the areas with higher cellularity without necrosis and estimated the number of cells for each field as described by Liu *et al.*³⁵ After that, we counted the number of typical apoptotic cells in 10 consecutive HPFs, as described by these authors, and calculated the apoptotic index, which was defined as the number of apoptotic cells in each 1000 cell.

Immunohistochemistry

The streptavidin–biotin peroxidase complex method was used for immunohistochemistry. Briefly, 4 µm sections were cut from paraffin wax blocks, dewaxed, and hydrated. Factor VIII related antigen (F-VIII) immunostaining was performed with antigen retrieval by means of pepsin digestion, at room

temperature for 30 minutes. Oestrogen receptor (ER) (Dako-patts, Glostrup, Denmark), p53 (Immunotech, Marseille, France), and MIB-1 (Novocastra, Newcastle, UK) immunostainings were performed, preceded by antigen retrieval with incubation in 10mM citrate buffer for 7.5 minutes at 750 W (domestic microwave), in a thermoresistant container. Distilled water and buffer were added every 1.5 minutes to the container to prevent drying during the incubation process. Immunostaining for c-erbB2 (Dakopatts), transforming growth factor α (TGF-α) (Serotec, Oxford, UK), and sialyl Tn (STn; Dakopatts) was carried out with no previous antigen retrieval step. Induced epitope retrieval using a target retrieval solution was used for COX-2. The slides were treated with 3% peroxide (H₂O₂), in methanol for 10 minutes to quench the endogenous peroxidase activity. ER, p53, c-erbB2, STn, TGF-α, and MIB-1 immunostaining was carried out as described previously.³⁶ Polyclonal antibodies for F-VIII (Dakopatts) and COX-2 (Santa Cruz Biotechnology, San Diego, California, USA) were applied to the sections at a dilution of 1/150 (with a 30 minute incubation at room temperature) and 1/10 with an overnight incubation at 4°C, respectively. The anti-COX-2 antibody is specific for COX-2, and does not crossreact with COX-1; it recognises an epitope mapping to the C-terminus of human COX-2.

Negative controls were carried out by omission of the primary antibody. As positive controls, sections from previously studied cases of breast cancer known to express ER, p53, MIB-1, c-erbB2, TGF-α, and STn were used. Sections from highly angiogenic breast carcinomas and colon carcinomas known to express COX-2 were used as positive controls for F-VIII and COX-2, respectively.

Evaluation of the immunohistochemical data

ER, p53, TGF-α, c-erbB2, MIB-1, and STn were evaluated as described previously.^{36, 37} COX-2 positivity was indicated by the presence of brown cytoplasmic staining. Immunoreactivity was evaluated in epithelial cells, in neighbouring stroma, and in endothelial cells. All cases with any unequivocal staining of the cytoplasm of neoplastic cells for COX-2 were considered positive.³³ Angiogenesis was evaluated by immunohistochemical staining of intratumoral microvessels for F-VIII. Any positively staining single cell, or cluster of cells, clearly separated from adjacent clusters and background, with or without lumen, was considered an individual vessel, as recommended in previous studies.^{38, 39} Areas of fibrosis, necrosis, and inflammation, in addition to vessels with a muscle wall, were excluded from the counting. Microvessels were counted in the three most vascularised areas in a 200× field (0.74 mm²) by four observers simultaneously. Because there was no significant difference between the highest count and the average vessel count in each case, we used the results obtained in the average vessel count in each case.

Western blotting analysis

Total protein was extracted from frozen tissues using the Tripure isolation reagent (Boehringer, Mannheim, Germany) and measured by spectrophotometry. Equal amounts of protein were denatured by boiling for two minutes, and then electrophoresed in a 10% polyacrylamide gel with a 5% stacking gel. After electrophoresis, proteins were electrotransferred to a Hybond nitrocellulose membrane (Amersham, Little Chalfont, Buckinghamshire, UK). Membranes were incubated with antibodies against COX-2 (Santa Cruz Biotechnology) and β actin (Santa Cruz Biotechnology) at a 1/2000 dilution overnight at 4°C. After several washing cycles the membranes were incubated with horseradish peroxidase conjugated secondary antibody at a 1/2000 dilution, for 45 minutes at room temperature. Western blotting protein products were visualised using the ECL chemiluminescence system (Amersham), after membrane exposure to an autoradiography film

Table 1 Cyclo-oxygenase 2 (COX-2) expression in tumour cells in relation to other clinicopathological features

Clinicopathological features	COX-2 expression in the neoplastic cells			p Value
	No. of cases	Positive	Negative	
Age in years (mean (SE))	46	49.8 (5.8)	57.4 (2.2)	0.82
Histological grade				0.16
I	8	0/8	8/8	
II	19	3/19	16/19	
III	16	5/16	11/16	
Nodal status				0.03
Positive	22	7/22	15/22	
Negative	20	1/20	19/20	
Transforming growth factor α				0.38
Positive	34	7/34	27/34	
Negative	11	1/11	10/11	
Oestrogen receptor content				0.46
Positive	27	4/27	23/27	
Negative	17	4/17	13/17	
p53 expression				0.82
Positive	20	2/20	18/20	
Negative	23	4/23	19/23	
c-erbB2 expression				0.39
Positive	11	3/11	8/11	
Negative	32	5/32	27/32	
Sialyl-Tn expression				0.02
Positive	14	6/14	8/14	
Negative	21	2/21	19/21	
Microvessel count (mean (SE))	46	66.6 (9.0)	44.6 (4.3)	0.03
Apoptotic index (mean (SE))		7.2 (1.3)	3.9 (0.5)	0.03
Mitotic count (mean (SE))	43	17.7 (2.3)	17.7 (2.7)	0.99
MIB-1 expression (mean (SE))	35	22.2 (4.8)	24.1 (4.2)	0.88

(Hyperfilm; Amersham). The highly invasive breast cancer cell line Hs578T, which is known to express COX-2, was used as a positive control⁴⁰ and β actin was used as the internal control.

Statistical analysis

Statistical analysis was carried out using a Statview program. Statistical differences between COX-2 expression and histological grade, node status, TGF- α expression, ER content, p53 expression, c-erbB2 expression, and STn expression were calculated using the χ^2 test. Analysis of variance (ANOVA) with Yates correction was used to compare COX-2 expression and mean values for age, proliferative index, mitotic counts, apoptotic index, and microvessel density. A value of $p < 0.05$ was considered significant. As a further validation of the apoptotic index and mitotic counts, we compared these two continuous variables with the MIB-1 proliferation index using Spearman's rank correlation.

To perform the disease free survival analysis, patient groups were defined according to positive or negative immunoreactiv-

ity for COX-2 and were compared for disease free survival using the Cox-Mantel non-parametric test. The test was performed unilaterally for all of the comparisons; the null hypothesis was that both groups were equal in terms of time under evaluation, and the alternative hypothesis was that one of the groups evolved better than the other.

RESULTS

Immunohistochemical analysis of breast tumour tissues for COX-2

Immunohistochemical staining of the formalin fixed, paraffin wax embedded tumour tissue revealed COX-2 cytoplasmic immunoreactivity in eight of the 46 patients with invasive ductal breast carcinoma studied. COX-2 expression was restricted to tumour cells in all the positive samples (fig 1A). Neighbouring stromal cells, such as fibroblasts and endothelial cells, showed no immunoreactivity for COX-2. Table 1 summarises the associations between COX-2 expression in

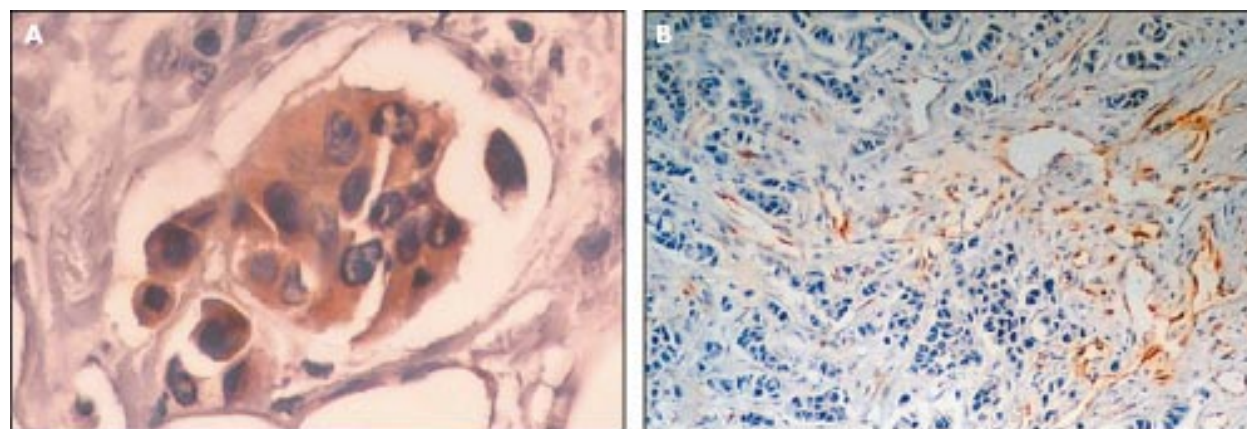


Figure 1 Immunohistochemical analysis of breast tumour tissue using antibodies to (A) cyclo-oxygenase 2 and (B) factor VIII related antigen to evaluate angiogenesis.

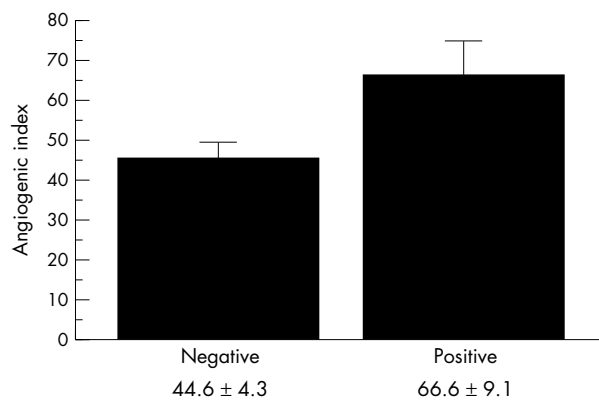


Figure 2 Association between cyclo-oxygenase 2 expression and angiogenic index (mean ± SE) evaluated by immunohistochemistry ($p = 0.03$).

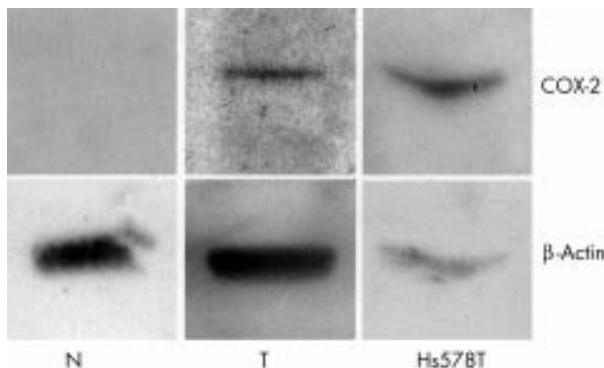


Figure 3 Western blotting analysis for adjacent normal tissue (N) and a breast tumour (T). The Hs578T breast cancer cell line was used as a positive control for cyclo-oxygenase 2 (COX-2) expression. Note the presence of a low intense band for COX-2 in the tumour and the absence of COX-2 in the adjacent normal tissue.

malignant cells and other clinicopathological features studied. Tumour expression of COX-2 was significantly associated with microvessels count evaluated by F-VIII expression (table 1; figs 1B,2). COX-2 expression was also significantly associated with lymph node metastases, apoptotic index, and STn expression in neoplastic cells. No significant relation was found between COX-2 immunostaining in tumour cells and the other clinicopathological characteristics analysed (table 1).

Western blotting analysis of COX-2 in breast tumour tissues and matched normal tissues

COX-2 expression was also analysed by western blotting in tumour specimens from 19 of the invasive ductal breast carcinoma frozen samples and two matched normal breast tissue samples. COX-2 expression was detected in eight of the 19 cases studied. In all cases in which COX-2 was detected by western blotting, immunohistochemistry was also positive for COX-2. In normal tissue there was no expression of COX-2. Figure 3 illustrates the western blotting results from a tumour and its matched normal tissue.

Mitotic counts and apoptotic index

The mean (SE) values of the mitotic counts in 10 HPFs and the apoptotic index in each 1000 cells were 17.74 (2.30) and 4.51(0.57), respectively. There was a highly significant correlation between mitotic counts and apoptotic index ($p < 0.0001$). In addition, an equally strong significant associ-

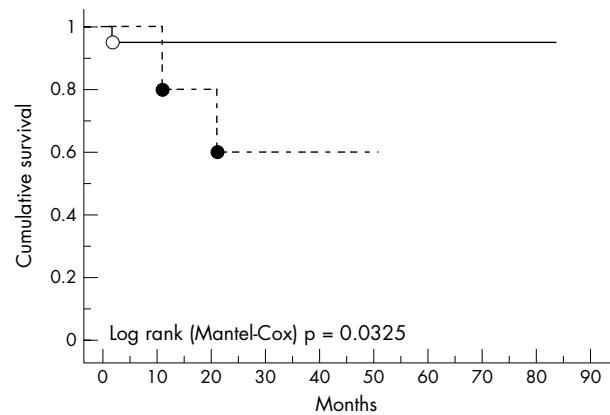


Figure 4 Cyclo-oxygenase 2 (COX-2) positive and COX-2 negative breast carcinomas plotted against disease free survival.

ation was found between the mitotic index and the MIB-1 proliferation index ($p < 0.0001$).

COX-2 positive cases showed a significantly higher apoptotic index ($p = 0.031$); however, no association was found between mitotic counts or MIB-1 and COX-2 expression ($p = 0.995$ and $p = 0.883$, respectively).

Disease free survival analysis

For the disease free survival analysis, we only included the 26 patients who attained a minimum 24 month follow up period. The relation between COX-2 expression and disease free survival is shown using Kaplan-Meier survival curves (fig 4). Using the log rank Cox-Mantel test, patients with breast cancer whose tumours were COX-2 positive had a significantly shorter disease free survival ($p = 0.0325$).

DISCUSSION

Our study revealed that the COX-2 protein was expressed in cases of human invasive ductal breast carcinoma, analysed by both immunohistochemistry and western blotting, whereas the COX-2 protein was undetectable in normal breast tissue. These results are in accordance with the data reported by Parrett and colleagues³² and Hwang *et al.*³³ Both these studies, which used different analytical methods, showed increased COX-2 expression in human breast carcinomas. Our figures (17.4% of the cases) were similar to those reported by Parrett *et al.*³² although they were lower than those described by Huang *et al.*³³ who showed COX-2 expression in 68.18% of breast cancer samples.

Although the role of COX-2 during tumorigenesis is not yet well defined, an association between tumour COX-2 expression and angiogenic status was seen in our study. Angiogenesis is required during tumorigenesis for the supply of oxygen and nutrients. At the same time, angiogenesis helps tumour cells to migrate through blood vessels and metastasise to distant organs. In our study, the expression of COX-2 was only seen in tumour cells and not in stromal or endothelial cells. However, the expression of COX-2 in tumour cells was related to increased endothelial cell proliferation. COX-2 is one of the enzymes that catalyses the synthesis of prostaglandins, which are local hormones mainly involved in the inflammatory response. Recently, several reports have suggested a role for prostaglandins in angiogenesis. Arakawa *et al* showed that ulcer healing was accelerated when prostaglandins are synthesised by COX-2, but not by COX-1.⁴¹ These findings emphasise the putative stimulation of angiogenesis by a COX-2 dependent mechanism. Accordingly, other studies suggest that prostaglandins exhibit vasoactive and mitogenic properties and are important mediators of increased vascular

permeability in the endometrium.⁴² COX-2 has also been proposed to reduce the adherence of tumour cells to the extracellular matrix, promoting angiogenesis and the development of metastasis. Similarly, COX-2 overexpression has been seen in several pathological states, such as cancer and rheumatoid arthritis, in which angiogenesis is strongly required. All these findings are in agreement with our results, suggesting that COX-2 may also play a role in angiogenesis. The involvement of COX-2 in angiogenesis has already been reported in other cancers, such as colorectal and gastric cancer.^{24, 25} However, to our knowledge this is the first study to suggest that COX-2 might also play a role in angiogenesis in breast cancer.

Despite the various reports of the involvement of COX-2 in angiogenesis, little is known with regard to its mechanism. Tsujii *et al* showed that tumours expressing COX-2 often produce VEGF and basic fibroblast growth factor, two growth factors associated with angiogenesis.²⁴ VEGF is a specific endothelial cell promoter because it acts by binding to its receptors (VEGFR-I and VEGFR-II) that exist in the cytoplasmic membrane of endothelial cells. VEGF is also called vascular permeability factor because of its ability to increase vessel permeability, which is important during both angiogenesis and inflammation processes. Because COX-2 and its products (prostaglandins) are mainly regarded as inflammatory factors, it is probable that they stimulate VEGF to induce permeability. COX-2 is overexpressed in several cancer types and it seems to upregulate angiogenesis; therefore, the use of COX inhibitors (such as non-steroidal anti-inflammatory drugs or preferentially specific COX-2 inhibitors) might be useful in antiangiogenic treatment.

"To our knowledge this is the first study to suggest that cyclo-oxygenase 2 might also play a role in angiogenesis in breast cancer"

We found no significant association between COX-2 expression and age, tumour grade, TGF- α , ER receptor content, p53, c-erbB2, or MIB-1. Despite the observations by Lui and Rose⁴³ in two human breast cancer cell lines, where COX-2 expression is regulated by hormonal status, no significant association between COX-2 and ER status was found in our series. This might be because of the low sensitivity of the immunohistochemical analysis performed or because of the fact that there are two ERs: ER α and ER β .⁴⁴ In our study, we only analysed ER α ; thus, our results may have been an underestimation. Although COX-2 is an inflammatory mediator, no association was found between COX-2 expression and tumour inflammatory infiltration (data not shown). Sawaoaka *et al*,⁴⁵ studying normal gastric epithelial cells, showed that COX-2 was induced by TGF- α and thereafter enhanced cell proliferation. In a previous study, we found that TGF- α was present in 75.1% of the breast cancer cases studied and was associated significantly with angiogenesis.⁴⁶ However, in that study we found no association between TGF- α and COX-2.

Despite the fact that COX-2 positive cases did not exhibit higher proliferative indices, as evaluated both by MIB-1 immunoeexpression and mitotic counting, we found a strong association between COX-2 expression and the apoptotic index. These findings provide further evidence for the putative role of COX-2 in the control of apoptosis in neoplastic cells of several types of human cancer,²¹ including human breast cancer cells.

In our present study, we also found an association between COX-2 expression, lymph node status, and STn expression. STn is a simple mucin-type carbohydrate antigen, which is also associated with lymph node metastases, as described previously by our group.³⁷

We found a significant association between COX-2 expression and a reduced disease free survival of patients with breast cancer in univariate analysis. In view of the small number of

Take home messages

- Cyclo-oxygenase 2 (COX-2) expression appears to be associated with angiogenesis, lymph node metastasis, and apoptosis in human breast cancer
- However, our results indicate that COX-2 expression in human breast cancer might be a late event in tumour progression and not involved in tumorigenesis
- Further studies with larger series of patients are needed to confirm the association with short disease free survival in patients with breast cancer

patients included in our study, these findings warrant larger studies with multivariate analysis to clarify the association of COX-2 and poor prognosis in patients with breast cancer.

In conclusion, we found a significant association between COX-2 expression, microvessel density, apoptotic index, STn expression, and lymph node status. These findings could indicate that COX-2 expression in human breast cancer might be a late event in tumour progression, in contrast to colorectal and gastric cancer, in which COX-2 plays a role in tumorigenesis and is considered to be an early event.

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