ORIGINAL ARTICLE

Overexpression of cyclo-oxygenase-2 is an independent predictor of unfavourable outcome in node-negative breast cancer, but is not associated with protein kinase B (Akt) and mitogen-activated protein kinase (ERK1/2, p38) activation or with Her-2/neu signalling pathways

K J Schmitz, R Callies, J Wohlschlaeger, R Kimmig, F Otterbach, J Bohr, H-S Lee, A Takeda, K W Schmid, H A Baba

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See end of article for authors' affiliations

Correspondence to: H A Baba, Institute of Pathology, University Hospital of Essen, D-45147 Essen, Hufelandstr 55, Germany; hideo.baba@medizin.uni-essen.de

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Background and aim: The production of prostaglandins is regulated by cyclo-oxygenases (COXs), which also have a role in tumour development and progression in various malignancies, including breast cancer. The mechanisms by which COX-2 contributes to unfavourable prognosis are still poorly understood. The association between expression of COX-2 and possible linked signalling pathways—namely, Akt, extracellular regulated kinases (ERK1/2), the stress-activated kinase p38 or Her-2/neu—is assessed in a series of 113 node-negative breast cancers.
Results: COX-2 was identified as an independent prognostic factor ($p=0.034$) in node-negative breast cancer by survival analysis. The lack of a relationship between COX-2 expression and activated Akt, Erk1/2, p38 and Her-2/neu was indicated by statistical analysis.
Conclusions: The prognostic effect of COX-2 expression on lymph node-negative breast cancer is confirmed—COX-2 is probably not regulated by HER-2, Akt, Erk1/2 or p38. Further studies are necessary for the elucidation of the signalling pathways responsible for the modification of COX-2 expression and the increased aggressiveness of breast cancers overexpressing COX-2.

Breast carcinoma is the most common malignancy in women. In the US, in 2005, about 211 000 cases of breast cancers were diagnosed, with 40 800 patients dying from the disease.[1] Besides commonly accepted prognostic markers such as lymph node status, tumour size and grade,[2] numerous other potential prognostic parameters have emerged. Among the potential prognostic parameters, cyclo-oxgenase (COX)-2 is of particular interest, as it may also offer the option of treatment with non-steroidal anti-inflammatory drugs (NSAIDs).[3] Cyclo-oxygenases regulate the synthesis of prostaglandins and are thus the major target of NSAIDs. Its two isoforms (COX-1 and COX-2) have different expression patterns, with COX-1 being expressed in a broad variety of tissues. COX-2 and its main product, prostaglandin E_2 (PGE_2), are inducible by growth factors and inflammatory stimuli. Additionally, COX-2 has been shown to participate in tumour development and progression.[4 5] Prostaglandins exert their effects locally in both autocrine and paracrine patterns. The effects of PGE_2 are mediated by a family of G protein-coupled receptors—namely, EP_1, EP_2, EP_3 and EP_4. The relevance of prostaglandins in breast cancer was studied by Bennett and Rolland more than 20 years ago—they found raised prostaglandin levels in breast cancer tissue and indicated that prostaglandin production may serve as a prognostic marker.[6–8]

Both COX-2 mRNA and protein levels were found to be raised in human breast cancer,[9 10] which was associated with decreased survival.[11 12] The application of NSAIDs was markedly associated with reduced development and progression of colonic tumours in patients with familial adenomatous polyposis (FAP),[13 14] whereas the data obtained on breast cancer are conflicting.[15 16]

The mechanisms by which COX-2 contributes to the poor prognosis have not been completely elucidated yet. COX-2 has been shown to contribute to both tumorigenesis and the malignant phenotype of tumour cells by different mechanisms, including (1) the inhibition of apoptosis by increased production of PGE_2, leading to increased expression of Bcl-2 and attenuation of nitric oxide (NO) signalling; (2) increased angiogenesis by increased PGE_2 production with subsequent vascular endothelial growth factor production; (3) increased invasiveness by overexpression of CD44; and (4) increased cell growth by activation of EP receptors.[17] The growth factor-mediated activation of the PI3-kinase/PKB (Akt) pathway is a key element of cellular survival and proliferation. Four phosphorylation sites (serine 124, threonine 308, threonine 450 and serine 473) have been identified at the PH domain of Akt. Activation of the Akt kinase requires phosphorylation at the serine 473 and threonine 308 sites. So far, the studies evaluating the potential prognostic value of the Akt kinase in breast cancer have yielded conflicting results.[18 19] Activation of the Akt kinase is markedly associated with reduced overall disease-specific survival.[20] St Germain and coworkers[21 22] suggested that the PI3-kinase–Akt pathway participates in

Abbreviations: COX, cyclo-oxygenase; ERK, extracellular regulated kinases; FAP, familial adenomatous polyposis; FISH, fluorescent in situ hybridisation; IHC, immunohistochemistry; MAPK, mitogen-activated protein kinase; NSAID, non-steroidal anti-inflammatory drug; pAkt, phospho-Akt; pERK, phospho-ERK; PGE_2, prostaglandin E_2; pp38, phospho-p38; SSC, sodium salt citrate

the regulation of COX expression. In addition to the serine–threonine kinase Akt, two members of mitogen-activated protein kinase (MAPK) signalling pathways are implicated in the expression of COX-2—namely, extracellular regulated kinases (ERK1/2) and stress-activated protein kinase p38.[23] A recent study identified stress-activated kinase p38 as an upstream regulator of COX-2 expression and showed over-expression of both phospho-p38 (pp38) and COX-2 in ductal carcinoma in situ.[24] Besides the Akt and MAPK signalling pathways, Her-2/neu has also been implicated in the regulation of COX-2.[25]

This immunohistochemical study assesses the possible association between COX-2 expression and the activation of putative signalling pathways, namely phospho-Akt (pAkt; serine 473 and threonine 308), phospho-ERK (pERK)1/2, pp38 or Her-2/neu, in a series of 113 node-negative breast cancers.

MATERIALS AND METHODS

Patients

This study comprised 117 female patients with breast cancer (mean age 61.9 years), who underwent surgery and further adjuvant treatment at the Clinic of Gynaecology (University of Duisburg-Essen, Essen, Germany) between 1989 and 1996. Clinical records and follow-up information (overall disease-specific survival) were available in all cases. Only patients with a negative lymph node status, which was confirmed on histological examination after standard axillary dissection, were included. All surgical material was fixed in 4% formalin and routinely processed. The tumours were classified according to the pathological tumour-node-metastasis system (6th edition) and graded according to Ellis and Elston.[26] During follow up, 17 of 117 patients died: 13 patients died from breast cancer, the remaining 4 patients died from either benign disease (n = 2) or concurrent cancer (n = 2) and were thus excluded from survival analysis. Statistical analysis was based on a median follow-up period of 7 years.

Immunohistochemistry (IHC)

Cyclo-oxygenase-2 and pAkt immunostaining

In this study, a monoclonal rabbit-anti-human COX-2 antibody (DCS, Hamburg, Germany) was used. Immunostaining was carried out on 5-µm-thick paraffin-wax sections with an automated staining device (DAKO Autostainer, Glostrup, Denmark). Antigen retrieval was carried out with 0.01 M citrate buffer at pH 6.1 for 20 min in a hot water bath (95°C). The primary antibody was incubated for 30 min at 1:250 dilution. Antibody was demonstrated with a commercially available antibody anti-mouse IgG detection kit (EnVision, DakoCytomation, Carpenteria, California, USA). Positive controls (colorectal carcinoma) were included in each staining series. Mouse immunoglobin replacing the primary antibody was used as a negative control.

In invasive carcinoma COX-2 was scored as follows: no immunoreactivity (−); positive staining independent of the amount of tumour cells (+). Immunostaining with pAkt (1/2/3) serine 473 and pAkt (threonine 308) was carried out with polyclonal rabbit anti-human antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA). Subsequent to antibody retrieval (as described earlier), the primary antibodies were incubated overnight in a humidified chamber at 4°C at a 1:2000 dilution (pAkt serine 473) and 1:5000 dilution (pAkt threonine 308). The alkaline phosphatase anti-alkaline phosphatase method was used for demonstration of the antibodies.

pAkt immunostaining was scored as the percentage of positively immunostained tumour nuclei by assessing 300 tumour cells at the invasive tumour front (as tumour aggressiveness is considered to be highest here). The percentage of pAkt serine 473-positive and threonine 308-positive tumour cell nuclei ranged from 7% to 87% and from 10% to 95%, respectively. Tumours with ⩾64% positive nuclei (mean value pAkt 473) and 61% (mean value pAkt 308) were classified as pAkt positive, respectively.

pERK1/2 IHC

Monoclonal phospho-p44/42 MAPK antibody (threonine 202/tyrosine 204; Cell Signalling Technology, Beverly, Massachusetts, USA) was used at a 1:100 dilution. Staining procedures were carried out as previously described.[20 27] Tumour cells with strong specific immunostaining, independent of the amount of stained cells, were scored as strongly positive (2+). Tumours exhibiting a detectable but faint immunostaining were scored as weak (1+), whereas tumours with a minimal, hardly detectable or missing staining pattern were classified as negative (0).

pp38 IHC

Monoclonal pp38 MAPK antibody (threonine 180/tyrosine 182; Cell Signalling Technology) was used at 1:1000 dilution. Antigen retrieval was carried out with 0.01 M citrate buffer at pH 6.1 for 45 min in a hot water bath (95°C). Antibody was demonstrated with a commercially available detection kit (ChemMate Detectin Kit, DakoCytomation). Positive controls (colorectal carcinoma) were included in each staining series. Mouse immunoglobin replacing the primary antibody was used as a negative control. Cytoplasmatic intensity of pp38 staining (1, absent; 2, low, moderate and strong) and nuclear pp38 staining (1, absent to low; 2, ⩽50% nuclear positivity; 3, >50% nuclear positivity) was evaluated. Addition of the intensity and proportion scores provided the final category score (negative, intermediate and strong) used for further statistical analysis.

Oestrogen receptor status

The oestrogen receptor status was determined with an anti-human oestrogen receptor α antibody (clone 1D5, DAKO, Glostrup, Denmark) as described previously.[20] Briefly, a tumour was regarded as receptor negative if none or <10% of the tumour cells showed weak or no nuclear immunostaining.

Fluorescent in situ hybridisation (FISH)

The *HER-2/neu* gene copy number was determined with the PathVysion HER-2/neu kit (Abbot GmbH, Diagnostica, Wiesbaden-Delkenheim, Germany) containing a mixture of SpectrumOrange-labelled *HER-2/neu* gene region and SpectrumGreen-labelled centromere region for chromosome 17. The *HER-2/neu* gene copy level was assessed as the ratio of *HER-2/neu* gene copies to chromosome 17 centromere copies. Sections embedded in paraffin wax (2 µm thick) were dewaxed for 3×5 min in xylene, dehydrated in graded ethanol series and washed in 2× sodium salt citrate (SSC; 1× SSC is 150 mM sodium chloride and 15 mM sodium citrate, pH 7). The sections were air dried, pretreated (80°C, 30 min) and digested with protease (37°C, 90 min) before hybridising with the PathVysion probe. Slide and probes were denatured simultaneously by incubation at 72°C for 1 min on a HYBrite hybridisation system (Vysis, Downers Grove, Illinois, USA). After incubating overnight in a dark humidity chamber at 37°C, the slides were washed for 2 min in 0.4×SSC/0.3% nitroprusside-40 at 72°C. The slides were then mounted in *p*-phenylenediamine dihydrochloride (Vysis), which contained *p*-phenylenediamine dihydrochloride as counterstain. The FISH analysis was carried out with an Nikon Eclipse 80i (Nikon GmbH, Düsseldorf, Germany). In every tumour cell nucleus that was considered for analysis,

the number of red and green signals was counted according to the manufacturer's guidelines, and the ratio of c-*erbB*-2 gene signals relative to chromosome 17 centromere signals per nucleus was calculated. Amplification of the c-*erbB*-2 gene was assumed at a ratio of >2.

HER-2 protein expression by IHC

The DAKO Hercept Test was used to detect the HER-2/neu protein expression (DAKO, number K 5204). Staining procedures were carried out according to the manufacturer's protocol. All IHC 2+ tumours were analysed with FISH to determine the level of the *HER-2/neu* gene copy. For statistical analysis, negative (DAKO score 0 and 1+; IHC 2+ cases lacking amplification) and positive (IHC 2+ cases with amplification and DAKO score 3+) groups were created.

Mitotic count

Mitotic count was calculated taking into consideration the actual area of 10 high-power fields (HPF) of the Nikon Eclipse E600 microscope. Mitotic count results were grouped as 0–8, 9–16 and >16 mitotic figures per HPF (field diameter 0.55 mm).[28]

Statistical analysis

All immunostaining was assessed in a blind-trial manner by two of the authors, who had no knowledge of the clinical outcome. All data were statistically analysed with SPSS V.12 for Windows. COX-2 and pAkt (serine 473) immunostains were evaluated by KJS and JB; pERK1/2 by KJS and JW; and pAkt (threonine 308) and pp38 by KJS and H-SL. In case the evaluators disagreed, slides were re-evaluated by both and a final decision was made. Interobserver agreement of categorical data (COX, pERK1/2 and pp38) was calculated with the κ coefficient; the κ value was interpreted using the commonly cited scale of Landis *et al*.[29] Interobserver agreement on COX-2, pERK1/2 and pp38 staining evaluation was substantial (κ = 0.618, 0.702 and 0.654, respectively). Interobserver agreement on continuous data was evaluated by using Pearson's correlation coefficient. Staining scores of pAkt serine 473 and threonine 308 yielded a significant interobserver concordance (Pearson's r = 0.605 and 0.677, respectively; $p<0.001$). The mean percentage of both pAkt stains was used for statistical analysis. Relationships between ordinal parameters were investigated by two-tailed χ^2 analysis. Pearson's correlation coefficient was used for detecting significant correlations among numerical parameters. Overall survival curves were estimated by the Kaplan–Meier method, and any differences in the survival curves were compared by the log rank test. For multivariate analysis, the Cox regression model was used. Overall, 95% confidence intervals were used throughout.

RESULTS

Analysis of HER-2 status

A total of 110 cases were analysed for HER-2/neu protein expression: 44 tumours were classified as negative (0), 38 as

Table 1 Clinicopathological data and cyclo-oxygenase-2 expression in node-negative breast cancers

Parameter investigated	COX-2 negative, n = 64 (56.68%)	COX-2 positive, n = 49 (43.4%)	p Value (χ^2 analysis)
Tumour type (n = 113)			NS
Invasive ductal	49 (43.4)	37 (32.7)	
Invasive lobular	12 (10.6)	3 (2.7)	
Mucinous/tubulo-lobular	2 (1.8)	6 (5.4)	
Others	1(0.9)	3 (2.7)	
Grading (n = 113)			0.004
Well	21 (18.6)	4 (3.5)	
Moderate	28 (24.8)	34 (30.1)	
Poor	15 (13.3)	11 (9.7)	
Oestrogen receptor status (n = 107)			NS
Positive	36 (33.6)	30 (28)	
Negative	25 (23.42)	16 (15)	
HerceptTest/FISH* (n = 110)			NS
Negative	52 (47.3)	39 (35.5)	
Positive	10 (9.1)	9 (8.2)	
Mitotic rate (n = 113)			NS
0–8	35 (31)	18 (15.9)	
9–16	15 (13.3)	21 (18.6)	
>16	14 (12.4)	10 (8.8)	
pAkt (308) (n = 95)			NS
Negative	20 (28.4)	30 (21.1)	
Positive	21 (22.1)	24 (25.3)	
pAkt (473) (n = 113)			NS
Negative	21 (21.1)	19 (31.6)	
Positive	43 (38.12)	30 (26.5)	
Tumour size (n = 113)			NS
pT1(a,b,c)	40 (35.4)	24 (21.2)	
pT2	23 (20.4)	25(22.1)	
pT3	1 (0.9)	0 (0)	
pERK1/2 (n = 110)			NS
Negative	8 (7.3)	6 (5.5)	
Intermediate	15 (13.6)	20 (18.2)	
Strong	38 (34.5)	23 (20.9)	
pp38 (n = 92)			
Negative	9 (9.8)	7 (7.6)	NS
Intermediate	25 (27.2)	22 (23.9)	
Strong	16 (17.4)	13 (14.1)	

COX, cyclo-oxygenase; ERK, extracellular regulated kinase; FISH, fluorescent in situ hybridisation; NS, not significant; pAkt, phospho-Akt; pERK, phospho-ERK; pp38, phospho-p38.
*FISH of all immunohistochemistry 2+ tumours was carried out. Cases without amplification were classified as negative.

negative (1+), 15 as weakly positive (2+) and 13 as strongly positive (3+). Of the 15 cases scored as weakly positive (2+), two showed a *Her-2/neu* gene amplification.

Immunohistochemical analysis of COX-2

In tumour cells COX-2 immunostaining was exclusively observed in the cytoplasm, with a granular staining pattern. Normal breast tissue constantly lacked COX-2 immunoreactivity. In contrast with the homogeneous positive staining pattern in colorectal cancer, a rather scattered distribution of COX-2 protein expression was observed in breast cancer specimens. Table 1 summarises the statistical correlation of the COX-2 status with clinicopathological parameters, as well as the other immunostaining results of this study. In χ^2-analysis COX-2 overexpression was significantly associated with a poorer differentiation grade ($p = 0.004$), whereas all other parameters investigated lacked significance. Figure 1 shows representative immunohistochemical COX-2 staining.

Immunohistochemical analysis of pAkt (threonine 308 and serine 473)

Immunostaining for pAkt (serine 473) was predominantly localised to tumour nuclei. Occasionally a specific faint cytoplasmatic pAkt staining was noticed, but was disregarded for calculation of immunohistochemical scores. Immunostaining for pAkt (threonine 308) was mainly located to the nucleus, with more intense cytoplasmatic staining than for pAkt (serine 473). Table 1 summarises the results of χ^2 analysis of pAkt expression and relevant clinicopathological parameters. Significant concordance between pAkt (serine 473) and pAkt (threonine 308) staining scores (Pearson's $r = 0.420$; $p<0.001$) was observed. Figure 2 shows representative immunohistochemical pAkt staining.

Immunohistochemical analysis of pERK1/2 and pp38

Both pERK1/2 and pp38 immunostaining exhibited a specific nuclear and cytoplasmatic staining pattern. Whereas normal breast tissue did not show any pERK staining, pp38 immunostaining was occasionally detected in normal breast tissue adjacent to invasive breast cancer. Table 1 summarises the results of pERK1/2 and pp38 immunostaining. No relationship was found between pERK1/2 or pp38 staining results and COX-2 expression.

Relationship of COX-2 and pAkt expression

χ^2 analysis showed no correlation between COX-2 expression and pAkt (threonine 308) and pAkt (serine 473) expression. No relationship was found between the number of positively stained tumour nuclei for pAkt (serine 473 and threonine 308) and COX-2 protein expression (fig 3), either.

Prognosis

COX-2 overexpression of the complete series was significantly associated with decreased overall survival ($p = 0.0191$; fig 4). Parallel analysis showed that histological grading ($p = 0.0010$), mitotic count ($p = 0.0393$) and the pAkt (serine 473) status ($p = 0.0190$) were significantly related to a decreased overall survival. A negative oestrogen receptor status tended to be associated with ($p = 0.076$) a worse prognosis (table 2). Increased tumour size (cut-off point 1.7 cm) was significantly associated with reduced overall survival in univariate analysis ($p = 0.0462$). If the cut-off point was, however, selected according to the tumour-node-metastasis classification, tumour size failed to reach statistical significance.

COX-2 and the relevant clinicopathological parameters (histological grading mitotic count and pAkt) were subject to

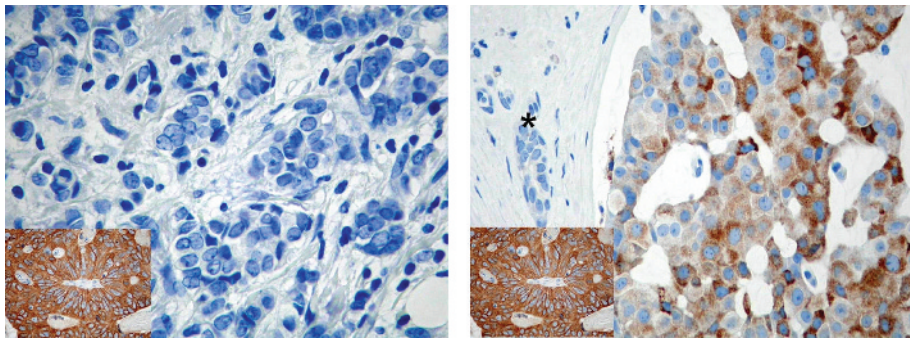

Figure 1 Representative immunohistochemical staining of cyclo-oxygenase-2 (COX-2) in node-negative breast cancers: breast carcinoma completely lacking COX-2 protein expression (left). Specific cytoplasmatic COX-2 expression (right). The complete lack of immunostaining in an adjacent normal breast duct (asterisk) is seen. Inset: strong COX-2 expression in a colorectal carcinoma serving as positive control. Original magnification ×400.

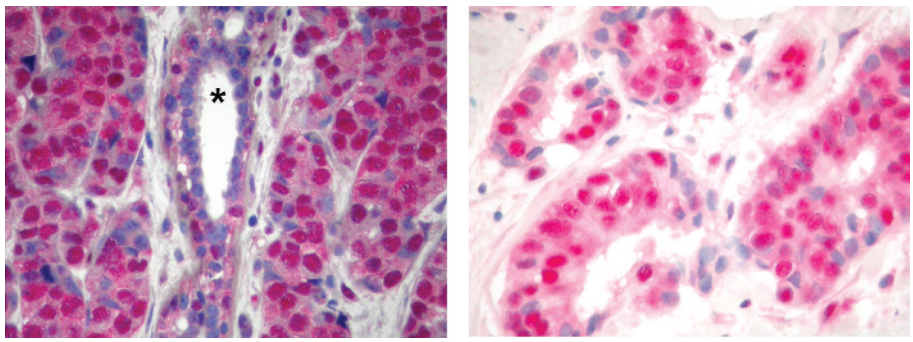

Figure 2 Representative immunohistochemical staining of two samples of breast cancer for phospho-Akt (pAkt; threonine 308; left) and pAkt (serine 473; right). Tumour cell nuclei mainly showed strong pAkt immunoreactivity; weak immunostaining was observed in the cytoplasm of tumour cells. The scattered positively stained nuclei are seen in a normal breast duct (asterisk). Original magnification ×400.

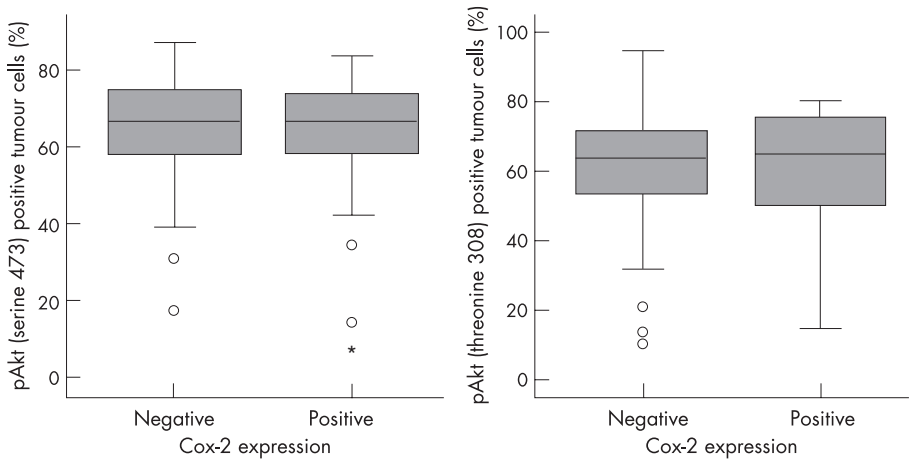

Figure 3 Box plots showing the relationship between cyclo-oxygenase-2 (COX-2) immunoexpression and the percentage of phospho-Akt (pAkt; serine 473 and threonine 308) positively stained tumour nuclei. Tumours with or without COX-2 expression exhibited equal amounts of pAkt tumour cells independent of the phosphorylation site. Open circles indicate outliers; asterisks indicate extreme values. Data were available for 95 cases (pAkt threonine 308) and for 113 cases (pAkt serine 473).

multivariate analysis; only COX-2 expression ($p = 0.034$) and histological grading ($p = 0.010$) were statistically associated with the overall survival of patients (table 3).

DISCUSSION

Novel markers defining node-negative high-risk patients who benefit from adjuvant treatment would be desirable, particularly in patients with node-negative breast cancer. This study on a series of 113 node-negative breast cancers showed an independent correlation of COX-2 expression with disease-specific overall survival in multivariate analysis, thus supporting previous reports describing a prognostic impact of COX-2 expression in node-negative breast cancer.[11 12] Against the background of the well-known COX-2 inhibiting effect of NSAIDs, recent studies have investigated the association of NSAID use and breast cancer risk. In fact, a reduction in risk with aspirin use was seen among patients with breast cancer with hormone receptor-positive tumours. These findings indicate that NSAIDs may serve as effective chemopreventive agents for breast cancer.[30]

Previous studies showed that COX-2 is upregulated in a variety of cancers—for example, colon, breast, lung, pancreas and oesophagus.[31–35] In cell culture and animal models, induction of COX-2 has been shown to promote cell growth, inhibit apoptosis and improve cell motility and adhesion.[36] The mechanisms underlying these multiple actions of COX-2 are, however, still largely unknown. The serine/threonine kinase Akt, which may function as an upstream activator or downstream effector of the *COX-2* gene, was found to regulate COX-2 transcription in human endometrial cancer cells by increased transcriptional activity of nuclear factor-κB. This resulted in an increased *COX-2* gene expression, suggesting that Akt was an upstream regulator.[21 22] Additionally, inactivation of glycogen synthase kinase-3β by Akt seems to play an important part in the ultraviolet-β induction of COX-2 transcription in human keratinocytes.[37] In contrast, a recently published study on hepatocellular cancer cells and human hepatocellular carcinoma tissue described the activation of Akt as a downstream signalling event induced by COX-2 overexpression.[38] Transfection of COX-2 expression vector in human hepatocellular cells resulted in improved phosphorylation of Akt, particularly at the phosphorylation site threonine 308.

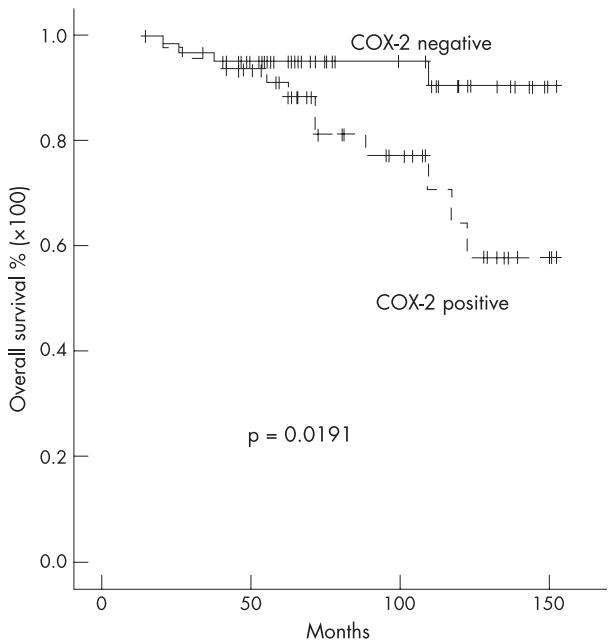

Figure 4 Kaplan–Meier survival plot of 113 node-negative breast cancers in relation to cyclo-oxygenase-2 (COX-2) protein expression. Log rank test: $p = 0.0191$.

Table 2 Univariate analysis (Kaplan–Meier, log-rank test) for prognostic significance of cyclo-oxygenase-2, histological grading, phospho-Akt (serine 473 and threonine 308), receptor status, HER-2/neu, mitotic count, tumour size and phospho-p38

Parameter	p
COX-2 (negative/positive)	0.0191
Histological grade	0.0010
pAkt (473) (negative/positive)	0.0190
pAkt (308) (negative/positive)	NS
ER status	NS
HER-2 status	NS
MC (0–8, 9–16, >16)	0.0393*
Tumour size	NS
pp38 (negative/intermediate/positive)	NS

COX, cyclo-oxygenase; ER, oestrogen receptor; MC, mitotic count; NS, not significant; pAkt, phospho-Akt; pp38, phospho-p38.

Table 3 Multivariate Cox regression analysis of disease-specific overall survival in 113 cases of node-negative breast cancers

Variable*	Relative risk	95% CI	p Value
COX-2	3.509	1.099 to 11.208	0.034†
Grading	5.537	1.244 to 24.532	0.025†
pAkt (473)	3.976	0.861 to 18.366	0.077
Mitotic count	0.774	0.278 to 2.156	0.624

COX, cyclo-oxygenase; pAkt, phospho-Akt.
*Coding of variables: pAkt was coded as 1, negative; and 2, positive. COX-2 was coded as 1, negative; and 2, positive. Mitotic count was coded as 1, 0–18; 2, 9–16; and 3, >16.
†Significant.

To our knowledge, this is the first study to analyse the association of COX-2 protein expression and activation (phosphorylation) of Akt in human breast cancers. Our immunohistochemical approach, however, failed to provide any evidence of a direct link between Akt phosporylation (both at serine 473 and threonine 308) and COX-2 overexpression.

Additional signalling pathways, including ERKs, the stress-activated kinase p38 and Her-2/neu, are thought to interact with COX-2. Cell line studies have shown that COX-2 expression can be stimulated by ERKs[23] and p38,[24] putting the ERK pathway and p38 pathway upstream of COX-2. A recent study on cells generated from human breast tissue explants found p38 to be responsible for COX-2 expression and established p38 as an upstream regulator of COX-2 in vitro.[24] Moreover, the same research group showed that activation of p38 and overexpression of COX-2 characterises ductal carcinoma in situ lesions and the nearby morphologically normal epithelium, whereas normal epithelium from disease-free breast tissue exhibited low pp38 levels. Interestingly, no direct correlation of COX-2 expression and pp38 levels in invasive breast cancers could be established in our study. As we did not analyse premalignant lesions of the breast, this is not surprising. Gauthier *et al* suggest that p38 may participate in an early event that contributes to a malignant phenotype in mammary epithelial cells. It is imaginable, however, that once a tumour has acquired a highly aggressive phenotype reflected by an invasive growth pattern, activation of p38 is not required anymore. This may explain the partly missing phospho-p38 (pp38) staining in COX-2-positive breast cancers. As for Akt, however, no relationship between activated Erk1/2 or p38 and COX-2 expression could be established in our series of node-negative breast cancers.

Furthermore, we showed that there was no correlation between Her-2/neu protein overexpression and COX-2. Recent studies on colon carcinoma and breast cancer cells provide evidence that Her-2/neu is implicated in the regulation of COX-2 overexpression.[25 39] In a clinical trial, a decreased HER-2 expression was observed in the rectal mucosa of patients with FAP after NSAID chemoprevention.[40] This suggests a regulatory effect of drug-related COX-2 expression on the HER-2 tyrosine kinase activity at least in patients with FAP. For breast carcinoma, a relevant regulatory role of Her-2/neu in COX-2 protein expression remains controversial. One study found a significant correlation between COX-2 and Her-2/neu,[11] whereas most other studies, including this study, did not find such a relationship.[12 34 41 42] We are aware that detection of changes in HER-2/neu by IHC is not the gold standard, especially in tumours scored 2+ by IHC (HerceptTest). In this subgroup, gene amplification was found in as many as 48% of cases.[43] To increase the significance of the statistical analysis of a potential correlation between HER-2 and COX-2 proteins, we determined the *HER-2/neu* gene copy level of all 2+ cases by FISH. We refrained from applying the FISH analysis to the complete series, as studies report an excellent correlation between FISH and IHC in 0, 1+ and 3+ scored tumours.[43] The lack of correlation with Her-2/neu, however, suggests that, unlike in colorectal cancer cells, type 1 receptor tyrosine kinase does not stimulate COX-2 expression in human breast cancer.

In conclusion, this study confirms the independent prognostic value of immunohistochemical COX-2 protein expression in node-negative breast cancer, thus offering an additional adjuvant therapeutic approach with COX-2 inhibitors. As we were unable to provide evidence for the regulation of COX-2 expression by HER-2, pAkt, pERK1/2 or pp38, further studies will be necessary to disclose the detailed signalling pathways responsible for the modification of COX-2 expression and the increased biological aggressiveness of breast cancers overexpressing COX-2.

Take-home messages

- Cyclo-oxygenase-2 (COX-2) protein expression is an independent prognostic predictor in node-negative breast cancer.
- COX-2 protein expression is probably not influenced by HER2, Akt, extracellular regulated kinase-1/2 or phospho-38.
- Further studies are necessary to elucidate the signalling pathways responsible for the modification of COX-2 expression in breast cancers.

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Authors' affiliations

K J Schmitz, J Wohlschlaeger, F Otterbach, J Bohr, H-S Lee, K W Schmid, Institute of Pathology, University Hospital of Essen, Essen, Germany
R Callies, R Kimmig, Department of Obstetrics and Gynaecology, University Hospital of Essen
A Takeda, Department of Internal Medicine, Gumma Paz Gakuen College, Gumma, Japan

Competing interests: RK, FO AND KWS are members of the West German Cancer Centre Essen (WTZE), Essen, Germany. KJS, RC, JW, RK, FO, KWS and HAB are members of the University Breast Cancer Centre Essen, Essen, Germany.

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