Expression of COX-2 and steroid converting enzymes in breast cancer

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Abstract. COX-2 is upregulated in many breast tumors, and one of the products of COX-2 is PGE2 that is suggested to upregulate aromatase through cAMP signaling in breast cancer. Although aromatase can increase the estrogen levels in tumors, 17ß-hydroxysteroid dehydrogenase (17HSD) activity is finally needed for the estrone/estradiol regulation. The aim of this study was to investigate if the protein expression of enzymes involved in estrogen synthesis shows covariation with the expression of COX-2. We also wanted to correlate these results with prognosis. We analyzed the expression of COX-2, aromatase, 17HSD1 and 17HSD2 with immunohistochemistry using tissue microarrays composed of 356 primary breast tumors. In the present study COX-2 was correlated to aromatase ($P<0.00001$), 17HSD1 ($P=0.0073$), and 17HSD2 ($P<0.00001$). Patients with ER positive tumors expressing low amounts of 17HSD2 had decreased breast cancer survival ($P=0.013$). Elevated expression of COX-2 and aromatase was more frequent among larger tumors ($P=0.017$ and $P=0.013$). COX-2 expression correlates with the levels of the examined steroid converting enzymes and may contribute to increased estrogen levels in the tumor. In breast cancer cells, the regulatory function of 17HSD2 could be lost, and in the present study patients with low or non-detectable levels of 17HSD2 had worse prognosis than had breast cancer patients with higher levels of the enzyme.

Introduction

In addition to the role of cyclooxygenase-2 (COX-2) in acute inflammation, recent work suggests that COX-2 derived metabolites are important during tumorigenesis such as pre-

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malignant proliferation, maintenance of tumor viability, growth, invasion and metastatic spread. Overexpression of COX-2 has been demonstrated in different human malignancies such as colon (1), lung (2), prostate (3), pancreas (4) and breast cancer (5). Several studies have demonstrated an enhanced expression of COX-2 mRNA and protein expression in breast carcinomas (6,7). Some studies reported a correlation between COX-2 expression and prognosis (8,9), whereas others did not find this association (10). The cyclooxygenases (COX-1 and COX-2) are responsible for the conversion of arachidonic acid to prostaglandins. COX-1 is constitutively expressed in most tissues and is responsible for maintaining psysiological processes. In contrast, COX-2 is induced by a wide spectrum of growth factors, oncogenes and cytokines in response to inflammation or neoplasia. A positive correlation between aromatase and COX-2 expression in breast cancer has been observed (11,12).

Estrogen is mitogenic to the breast and plays an important role in the growth of hormone-dependent breast cancer. The source of estradiol differs between pre- and postmenopausal women. Before menopause, the majority of estradiol is synthesized by the ovaries, and among postmenopausal women the cessation of ovarian estradiol production leads to a decrease in plasma estradiol levels. However, the levels of estradiol in human breast tumors among postmenopausal women are much higher than the estradiol level in plasma (13). The mechanisms responsible for maintenance of high estradiol concentrations are not completely defined but are likely to involve local production of estradiol by the tumor itself. Androstenedione, produced primarily by the adrenals, is converted to estrone by aromatase in extra glandular tissue. Estrone can also be conjugated by sulfatase into estrone sulfate to form a slowly turning over storage pool, with a potential for back conversion to estrone. Levels of the sulfatase enzyme are actually higher in breast tumors than the levels of aromatase, however, the affinity for the substrate is lower.

Even if aromatase can increase the estrone level in breast tumors, 17ß-hydroxysteroid dehydrogenase (17HSD) activity is finally needed for the estradiol/estrone regulation. The 17HSD1 enzyme uses NADPH as a cofactor and catalyzes the interconversion of the weak estrogen, estrone (E1), to the biologically more potent estradiol (E2). 17HSD2 uses NAD+
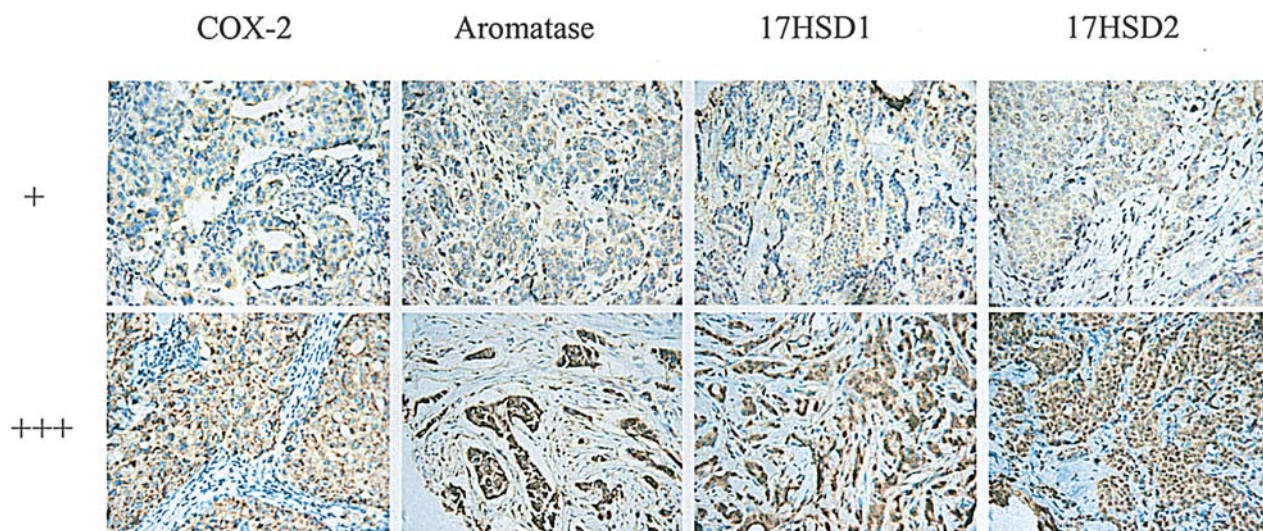
Figure 1. The immunostaining of COX-2, aromatase, 17HSD1, and 17HSD2 was scored as negative (-), weak (+), moderate (++), and strong (+++). Examples of tumors exhibiting weak and strong staining, respectively, are shown.

as a cofactor and catalyzes the oxidation of testosterone and estradiol to form androstendione and estrone, respectively (14). Previous studies have reported on the presence of multiple members of the 17HSD-family in human breast tumors, including types 3, 4 and 5 (15), although types 1 and 2 seem to be the principal enzymes involved in reductive and oxidative activity in breast cancer, respectively (16). In previous studies we have found that the expression of both 17HSD1 and 17HSD2 may have prognostic importance in breast cancer (17,18).

The aim of this study was to investigate if the expression of enzymes involved in estrogen synthesis shows association with the expression of COX-2 in breast tumors. We also correlated these results to prognosis.

Materials and methods

Patients. The patient series consisted of 356 breast cancer cases treated in Stockholm, Sweden, in 1977-1990. All patients had primary breast cancer and were postmenopausal at the time of diagnosis. All patients were lymph node negative and had a tumor diameter ≤30 mm. After surgery the patients were randomized to tamoxifen treatment or no endocrine treatment. The median follow-up time was 17 years. The study was approved by the regional ethics committee at Karolinska Institute.

Breast cancer tissue microarray (TMA). Representative tissue blocks were selected as donor blocks for the TMA. Sections were cut from each donor block and stained with hematoxylin and eosin. From these slides three morphologically representative regions were chosen (when possible) in each of the 356 tumor samples. Three cylindrical core tissue specimens (diameter 0.8 mm) were taken from these areas in each case and mounted in a recipient block, with a total amount at most of 243 cores/block. The tissue microarrays were constructed using a manual arrayer (Beecher Inc., WI,

USA). Six tissue array blocks were prepared, each containing 66-243 tumor sample cores. Liver samples were included on each tissue block as a control.

Immunostaining for COX-2, aromatase, 17HSD1 and 2 protein expression. TMA blocks were cut with a microtome into 4-μm sections and mounted on frost coated glass slides. The TMA sections were deparaffinized in xylene, rehydrated and thereafter rinsed in distilled water. Antigen retrieval was performed by placing the slides in an oven (78˚C) in citrate buffer (pH 6.0) for 20 h. The slides were incubated in 3% H_2O_2/methanol for 15 min to block the endogenous peroxidase activity. Thereafter, the sections were incubated with the COX-2 monoclonal antibody (160112; Cayman Chemicals Co., Ann Arbor, MI) in 1:250 dilution for 24 h at 4˚C, followed by incubation with a secondary antibody complex, EnVision HRP, anti-mouse (Dako, Denmark) for 25 min. After that, 3,3-diaminobenzidine tetrahydrochloride (DAB)/H_2O_2 was used for 8 min to visualize the immunoreaction, and finally the slides were counterstained with hematoxylin, dehydrated and mounted. The immuno-staining procedure with antibodies against aromatase (Nordic Biosite AB, Täby, Sweden), 17HSD1 and 17HSD2 (a kind gift from Professor F. Labrie and Professor V. Luu-The, Quebec, Canada) followed the same protocol as that for COX-2 immunostaining. However, these were all polyclonal (secondary antibody; Envision HRP anti-rabbit, Dako) and the dilutions were: aromatase 1:400, 17HSD1 1:2500, and 17HSD2 1:2000. Out of 356 tumors, approximately 19% were either detached during the preparation of the specimens or did not contain a sufficient number of tumor cells.

Evaluation of immunohistochemical staining. The slides were evaluated independently by two investigators (CG and OS) without knowledge of clinicopathological information. The intensity was classified as negative, weak, moderate or strong in tumor cells. In cases with different scoring results, a consensus score was reached after re-evaluation. In some

Table I. Correlation between COX-2, aromatase, 17HSD1 and 17HSD2 protein expression.

	Aromatase		17HSD1		17HSD2	
	- n (%)	+ n (%)	- n (%)	+ n (%)	- n (%)	+ n (%)
COX-2						
-	98 (63)	57 (37)	83 (56)	64 (44)	104 (66)	53 (34)
+	42 (41)	60 (59)	42 (44)	53 (56)	46 (48)	50 (52)
		P=0.00052		P=0.063		P=0.004
Aromatase						
-			85 (65)	45 (35)	95 (71)	38 (29)
+			38 (36)	67 (64)	45 (41)	65 (59)
				P=0.00001		P<0.00001
17HSD1						
-					81 (68)	39 (32)
+					52 (46)	61 (54)
						P=0.0093

statistical analysis, negative and weak cases were considered as a group with low expression levels, and cases with moderate and strong staining as a group with high levels.

Results

Association of COX-2 staining with aromatase, 17HSD1 and 17HSD2 staining. Immunoreactivity of COX-2 was evaluated in 284 breast carcinomas, of which 17.5% showed negative, 44.5% weak, 28% moderate and 10% strong staining. COX-2 immunoreactivity was localized exclusively to the cytoplasm of the tumor cells, whereas the stroma was negative. Expression of aromatase was successfully evaluated in 270 breast carcinomas, of which 9% showed negative, 47% weak, 38% moderate and 6% strong staining. Aromatase immunoreactivity was localized to the cytoplasm and in some cases to the stroma surrounding the tumor cells.

In 253 tumors the protein expression of 17HSD1 was assessable. The expression of 17HSD1 was negative in 8%, weak in 44%, moderate in 39.5% and strong in 8.5%. The immunoreactivity of 17HSD1 was predominantly observed in the cytoplasm. Among the 265 tumors analyzed, the 17HSD2 protein expression was negative in 16%, weak in 42.5%, moderate in 36%, and strong in 5.5%. 17HSD2 immunoreactivity was localized to the cytoplasm of the tumor cells, and the surrounding stroma was negative. Examples of weak and strong immunostaining for the different proteins are shown in Fig. 1.

When divided into four groups, COX-2 protein expression correlated to aromatase (P<0.00001), 17HSD1 (P=0.0073) and 17HSD2 (P<0.00001). In the subsequent analysis the expression levels were divided into two groups, high/+ (scores moderate/strong) or low/- (scores negative/weak) (Table I). The expression of aromatase correlated significantly with 17HSD1 (P=0.00001) and 17HSD2 (P<0.00001).

Associations with clinicopathological data. Elevated expression of COX-2 was significantly more frequent among larger tumors (P=0.017). No significant association was found between COX-2 protein expression and estrogen receptor (ER) status. Moreover, high aromatase expression was associated with large tumors (P=0.013), but no association was found between aromatase and ER-status (Table II).

Prognostic value of COX-2, aromatase, 17HSD1 and 17HSD2. The prognostic importance for the different enzymes was investigated among ER-positive patients. There was no significant difference in distant recurrence-free survival or breast cancer related survival between patients with COX-2 positive and COX-2 negative breast carcinomas (Table III). No prognostic difference was seen among patients with different expression for aromatase or 17HSD1. However, patients with ER positive breast tumors with low levels of 17HSD2 had a worse prognosis, both concerning distant recurrence (P=0.055) and breast cancer related death (P=0.013). Kaplan-Meier curves for distant recurrence-free survival and breast cancer related survival are depicted in Fig. 2. The prognostic value for 17HSD2 remained significant in a multivariate analysis with regard to breast cancer specific survival (Table IV). Among ER negative patients no significant prognostic value was detected with different expression levels of the enzymes.

Discussion

In the present study COX-2 correlated to aromatase as well as to 17HSD1 and 17HSD2. The significant association between COX-2 and aromatase is in line with earlier studies. Brodie *et al* (11) showed a strong positive correlation between aromatase and COX-2 expression in breast cancer, and Brueggemeier *et al* (12) have shown a strong association between mRNA levels of COX-2 and aromatase (12). In breast tumors the regulation of aromatase expression is associated with a switch in the promoter region utilized in gene expression. PGE2 (as a result of COX-2 activity) is a known stimulant of cAMP in breast cancer cells, and is suggested to be important in the switching of aromatase promoters (19). If these mechanisms

Table II. Expression of COX-2, aromatase, 17HSD1 and 17HSD2 in relation to other characteristics.

	COX-2		Aromatase		17HSD1		17HSD2	
	- n (%)	+ n (%)	- n (%)	+ n (%)	- n (%)	+ n (%)	- n (%)	+ n (%)
Tumor size								
<20 mm	133 (65)	71 (35)	115 (60)	76 (40)	93 (51)	89 (49)	108 (56)	86 (44)
≥20 mm	36 (49)	37 (51)	31 (43)	41 (57)	34 (53)	30 (47)	43 (66)	22 (34)
		P=0.017		P=0.013		P=0.78		P=0.14
ER status[a]								
ER⁻	30 (54)	26 (46)	23 (44)	29 (56)	23 (47)	26 (53)	33 (62)	20 (38)
ER⁺	119 (62)	74 (38)	106 (57)	80 (43)	87 (50)	87 (50)	102 (57)	77 (43)
		P=0.28		P=0.10		P=0.70		P=0.49
Tamoxifen								
No	80 (57)	60 (43)	72 (54)	62 (46)	64 (52)	60 (48)	73 (57)	56 (43)
Yes	95 (66)	49 (34)	78 (57)	58 (43)	68 (53)	61 (47)	82 (60)	54 (40)
		P=0.13		P=0.55		P=0.86		P=0.59

[a]ER⁺, ≥0.05 fmol/μg DNA.

Table III. Univariate analysis of distant recurrence and breast cancer related mortality rate in relation to COX-2, aromatase, 17HSD1 and 17HSD2 among ER positive patients (RR, rate ratio).

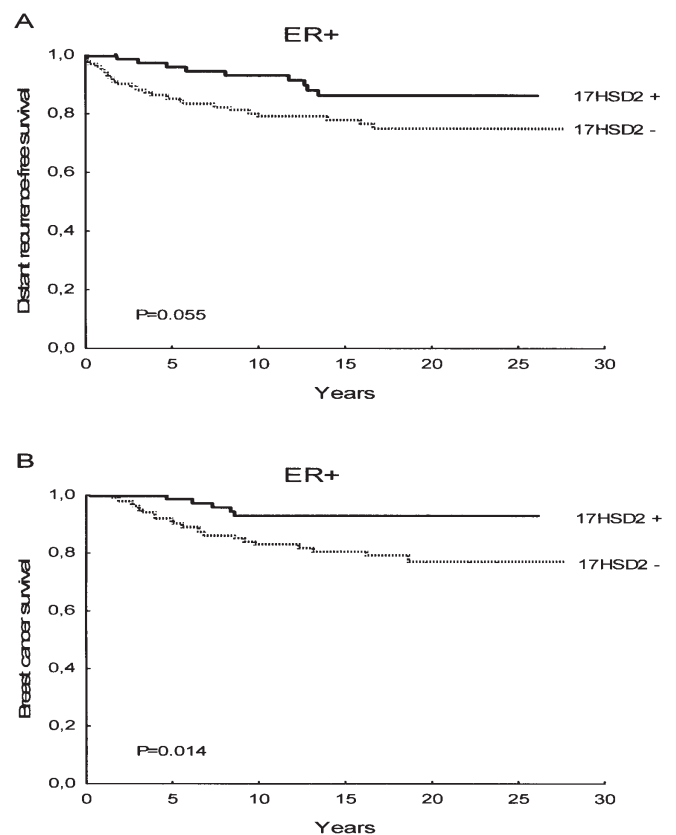|	No. of patients	Distant recurrence		Breast cancer death	
		RR	Significance	RR	Significance
COX-2					
-	119	1.0		1.0	
+	74	1.66	P=0.13	1.20	P=0.63
Aromatase					
-	106	1.0		1.0	
+	80	1.43	P=0.28	0.95	P=0.90
17HSD1					
-	87	1.0		1.0	
+	87	0.89	P=0.73	0.92	P=0.83
17HSD2					
-	102	1.0		1.0	
+	77	0.48	P=0.061	0.31	P=0.02

Figure 2. Distant recurrence-free survival (A) and breast cancer survival (B) among estrogen receptor positive patients with decreased and high protein expression of 17HSD2 (n=102 and n=77, respectively).

are important in breast cancer, a correlation between COX-2 and aromatase activity might be expected. Hovewer, cytokines such as IL-6 and TNFα have also been detected to increase aromatase and 17HSD1 activity (20). Miyoshi *et al* (21) have shown that intratumoral aromatase mRNA levels significantly decrease after chemotherapy, suggesting that antitumor activity of chemotherapy is mediated, at least in part, through suppression of intratumoral E2 synthesis. They further suggest that aromatase levels are regulated predominantly by TNFα and not by IL-6 or COX-2.

. Multivariate analysis of distant recurrence and
cer related mortality rate among ER positive patients
in relation to 17HSD2 and other variables (RR, rate ratio).

	No. of patients	Distant recurrence		Breast cancer related death	
		RR	Significance	RR	Significance
17HSD2					
-	101	1.0		1.0	
+	76	0.5	P=0.078	0.33	P=0.027
Tamoxifen					
No	80	1.0		1.0	
Yes	97	0.39	P=0.010	0.35	P=0.013
Tumor size					
≤20 mm	140	1.0		1.0	
21-30 mm	37	2.4	P=0.019	2.9	P=0.0077

Aromatase, 17HSD1 and 17HSD2 are involved in the local
regulation of estrogen (E1) and estradiol (E2). It has previously
been shown that in the normal breast tissue the oxidative path-
way (E2→E1) dominates, whereas in malignant breast tumors
the reductive pathway (E1→E2) is dominant (22). In the present
study there was a significant correlation between COX-2 and
17HSD1 and between COX-2 and 17HSD2. This has not pre-
viously been described and one possible explanation to this
result may be that enzymes involved in estrogen synthesis are
simultaneously upregulated. Sasano et al (23) found a
significant correlation between 17HSD1 and aromatase
among invasive lobular breast carcinomas. In the present
study we confirm the correlation between these enzymes in
breast tumors.

Surprisingly, 17HSD2 showed positive correlation with
all of the other enzymes (Table I). However, this may not
necessarily reflect that all of the enzymes are coregulated.
Concidering alternative mechanisms behind increased
estradiol levels, it might as well reflect a pattern with
17HSD2 positive tumors that more likely might be over-
expressing aromatase and 17HSD1, and 17HSD2 negative
tumors with normal aromatase and 17HSD1 levels.

In a recent study Oduwole et al (24) described that patients
with breast tumors expressing high levels of 17HSD1 mRNA
had shorter disease-free and overall survival, and they suggest
17HSD1 as an independent prognostic marker regardless of
ER status. Principally in line with their results we found that
high levels of 17HSD1 or low levels of 17HSD2 predict
recurrence among ER positive patients (17,18). We have
previously detected amplification of the gene coding for
17HSD1 in breast cancer, and among ER positive, tamoxifen
treated patients, this was associated with decreased breast
cancer survival (25). In the present study however, we were
not able to detect a prognostic value of 17HSD1.

COX-2 has been described as a marker in breast cancer
for more aggressive and less differentiated tumors. Ristimäki

et al (8) analyzed the COX-2 protein expression in 1567
invasive breast cancers with immunohistochemistry. Elevated
expression of COX-2 was observed in about 40% of the breast
tumors, and was significantly associated with decreased distant
recurrence free survival, negative hormone receptor status,
high proliferation grade and high p53 expression. O'Connor
et al (26) showed that COX-2 expression correlated with
treatment failure and diminished survival among women who
underwent mastectomy and chest wall radiotherapy. They also
found that the level of COX-2 expression was higher among
younger patients with invasive breast cancer. On the
contrary, Kelly et al (27) described a lack of prognostic effect
of COX-2 in primary breast cancer on short-term follow-up.
In the present study we did not find any significant difference
in prognosis among COX-2 positive or negative patients.
However, all patients in our study were postmenopausal and
lymph node negative.

In the present study there was a significant difference in
prognosis in relation to protein expression of 17HSD2 among
ER positive patients. Patients who had a low or undetectable
protein expression of 17HSD2 had a significantly higher risk
of breast cancer related death. This strongly supports our
previous findings, that low levels of 17HSD2 mRNA were
associated with a poor prognosis (17,18). It has been suggested
that 17HSD1 plays a more important role in breast cancer than
17HSD2 (23,24). However, the relative importance of these
enzymes may depend on tumor stage, and as mentioned
before the patients in the present study were all lymph node
negative. The loss of 17HSD2 expression could result in a
significant increase of the more biologically active E2. The
gene encoding 17HSD2 is located at 16q24 and loss of hetero-
zygosity (LOH) at this site is a frequent and early event in
breast cancer (28). Moreover, in prostate cancer LOH at 16q
is common, and Elo and colleagues (29) discussed whether
activity of 17HSD2 protects prostatic epithelial cells from
excessive androgen action and reduces the proliferative pressure
on prostatic cells. Significantly decreased mRNA expression
of 17HSD2 in prostate cancer as compared to normal prostate
tissue has also been reported (30).

Adjuvant tamoxifen therapy significantly improves the
survival of ER positive breast cancer patients. Recent clinical
trials have shown that the recurrence-free survival is further
increased with the use of aromatase inhibitor, but biological
markers for selecting patients for different kind of endocrine
treatment remain to be established. We hypothesize that the
expression levels of steroid converting enzymes might predict
the efficiency of these treatments.

We found that COX-2 was correlated to the expression of
enzymes involved in estrogen synthesis. As earlier mentioned,
COX-2 is supposed to upregulate aromatase through PGE2
and cAMP. Furthermore, it has been described that 17HSD1
is upregulated by cAMP in rat granulosa cells (31). If this is
true in breast epithelial cells, COX-2 could in part be respon-
sible for the high levels of E2 in breast cancer tissue, through
upregulation of both aromatase and 17HSD1. In the normal
breast, 17HSD2 will inactivate E2 to the less potent E1, but
in the malignant cells, the regulatory function of 17HSD2 might
be lost. In the present study we show that patients with low
or non-detectable levels of 17HSD2 had a significantly worse
prognosis than had patients with normal levels of the enzyme.

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