[CANCER RESEARCH 61, 8448–8451, December 1, 2001]

Abnormal Expression of 17β-Hydroxysteroid Dehydrogenases in Breast Cancer Predicts Late Recurrence[1]

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ABSTRACT

The 17β-hydroxysteroid dehydrogenase (17β-HSD) enzymes are involved in the interconversion of biologically active and inactive sex steroids and are considered to play a critical role in the *in situ* metabolism of estrogen, especially in estrogen-dependent breast cancer. The gene encoding 17β-HSD type 2 is located at 16q24.1-2, and earlier studies have shown that allelic loss in this region is an early and frequent event in breast cancer progression. Recurrence of hormone-dependent breast cancer frequently occurs several years after the primary treatment. The aim of this study was to investigate whether the expression of 17β-HSD types 1 and 2 differs in tumors from patients with late relapses (>5 years) compared with controls without recurrence after long-term follow-up. Using real-time reverse transcription-PCR, we found that the normal mammary gland expressed both 17β-HSD types 1 and 2, whereas the tumors frequently lacked detectable levels of type 2. Only 10% of the estrogen receptor-positive tumors expressed type 2, whereas 31% of the ER-negative tumors did so ($P = 0.031$). In a case-control series of 84 patients, a high level of 17β-HSD type 1 indicated increased risk to develop late relapse of breast cancer (odds ratio, 3.0; 95% confidence interval, 1.0–12.6; $P = 0.041$), whereas retained expression of type 2 indicated decreased risk (odds ratio, 0.25; 95% confidence interval, 0.05–1.2; $P = 0.050$). In multivariate analysis of the estrogen receptor-positive patients, the absence of 17β-HSD type 2 combined with a high expression of type 1 showed prognostic significance ($P = 0.016$) in addition to DNA aneuploidy ($P = 0.0058$), whereas progesterone receptor status did not ($P = 0.71$). These findings suggest that abnormal expression of 17β-HSD isoforms has prognostic significance in breast cancer and that altered expression of these enzymes may have importance in breast cancer progression.

INTRODUCTION

Estrogens play a predominant role in the regulation of cell growth and differentiation of the normal mammary gland as well as in hormone-sensitive breast carcinomas. Most breast carcinomas are detected after menopause, and despite a low degree of ovarian estrogen production and a low level of serum estrogen these tumors show a high *in situ* production of estrogens (1). This suggests that most breast cancers have an enzyme system efficient enough to produce active estrogens *in situ* from circulating precursors. Enzymes modulating local steroid availability seem to play an important role in the progression of breast cancer. Among these are isoforms of 17β-HSD,[4] which control the final step in the formation of androgens and estrogens (2). The 17β-HSD type 1 enzyme uses NADPH as a cofactor and catalyzes the interconversion of the weak estrogen E1 to the biologically more potent E2. 17β-HSD type 2 uses NAD^+ as a cofactor and catalyzes the oxidation of testosterone and E2 to form androstenedione and E1, respectively (3, 4). 17β-HSD types 1 and 2 thus cooperate to regulate the tissue level of the more potent E2; therefore, examining the expression of these enzymes is essential in an attempt to better understand the local regulation of estrogenic actions in human breast carcinoma.

A few immunohistochemical studies of 17β-HSD type 1 in human breast carcinoma have been reported, but the relation to prognosis and clinical parameters are still not clear (5–7). One of the studies suggested that 17β-HSD type 1 plays an important role in hormone-dependent breast carcinomas, whereas 17β-HSD type 2 was not detected in any of the tumors (7). Several studies have reported on the presence of multiple 17β-HSD isoenzymes in human breast cancer cells, including types 3 and 4 (8–10). In the normal glandular epithelium of the breast, no correlation was found between expression of type 4 and oxidative activity, whereas types 1 and 2 were evenly expressed and reflected reductive and oxidative 17β-HSD activity, respectively (9).

Although many patients with hormone-independent breast cancer relapse within the first few years after surgery, patients with ER-positive tumors more frequently have a recurrence much later. The aim of this study was to investigate the mRNA expression of 17β-HSD types 1 and 2 in tumors from a series of 42 patients with late relapses (>5 years) and 42 controls without recurrence. The controls were matched for tumor stage, patient age, and treatment, and the expression levels of the enzymes were related to the levels found in the normal mammary gland.

MATERIALS AND METHODS

Patients. We analyzed tissue from excised primary breast tumors of 84 women treated in the health care region of southeast Sweden between 1984 and 1991. All patients had primary breast cancer without distant metastasis at time of diagnosis. The mean age of the patients was 63 years. After surgery, the tumor samples were stored in a freezer (−70°C) until RNA extraction was performed. The patients consisted of two groups, each comprising 42 patients. In one group, all of the patients relapsed with distant metastasis 5 years after surgery or later, whereas for the patients in the other group (controls), no relapses had been registered during a median follow-up period of 13 years. The control patients were matched for tumor size, the number of positive lymph nodes, patient age, and tamoxifen treatment. In both groups, 35 women (83%) received adjuvant tamoxifen. We also examined the expression of 17β-HSD types 1 and 2 in normal breast tissue. Total RNA from a pool of eight normal human breast tissue samples was purchased from Clontech (Palo Alto, CA).

ER and PgR content was measured with enzyme immunoassays (Abbott Laboratories, Chicago, IL). Samples with concentrations ≥0.3 fmol/μg of DNA were classified as positive. Like the receptor assays, DNA flow cytometry was performed in clinical routine practice. A tumor with a DNA histogram showing more than one G_1 peak was classified as DNA aneuploid; otherwise it was classified as DNA diploid. A cutoff threshold of 10% was used to classify tumors as having low or high S-phase fractions.

RNA Extraction. Frozen breast tumor tissue (30 mg) was homogenized in a microdismembrator (B. Brown, Melsungen, Germany), and total RNA was extracted with the SV Total RNA Isolation System (Promega, Madison, WI). The purified RNA was stored at −70°C, and the RNA content was determined by spectrophotometry.

Received 5/21/01; accepted 10/2/01.

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[1] This study was supported by grants from the Swedish Cancer Society and the Research Council of Southeast Sweden (FORSS).

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[4] The abbreviations used are: 17β-HSD, 17β-hydroxysteroid dehydrogenase; E1, estrone; E2, estradiol; ER, estrogen receptor; PgR, progesterone receptor; LOH, loss of heterozygosity.

cDNA Synthesis. Total RNA (500 ng) was reverse-transcribed in a final volume of 20 μl, using the Gibco BRL kit (Life Technologies, Inc., Stockholm, Sweden) with following concentrations: 1× PCR buffer, 5 mM $MgCl_2$, 0.5 mM deoxynucleotide triphosphates, 2.5 μM random hexamers, 10 mM DTT, and 0.5 μl of Superscript reverse transcriptase (Life Technologies, Inc.). The thermal conditions used were as follows: 20°C for 10 min, 42°C for 50 min, 99°C for 5 min, and 5°C for 5 min. The samples were stored at 4°C until analysis. For every sample, 500 ng of RNA were also used for a control preparation that contained all reagents except the reverse transcriptase.

Primers and Probes. We used the computer program Primer Express (PE Applied Biosystems, Foster City, CA) to design primers and probes that recognized human 17β-HSD type 1 and 2 cDNA sequences. We conducted Blast searches (GenBank) to confirm the specificity of nucleotide sequences chosen for the primers and probes and the absence of DNA polymorphism. To avoid detection of contaminating genomic DNA, the probe was placed at the junction between two exons. In these cases we placed the probe between exons 1 and 2. The primer and probe sequences are presented in Table 1. Both primers and probes were purchased from PE Applied Biosystems, as were the primers and probe for *β-actin*, which was used as endogenous control gene. The specificity of the amplified products was also tested using a standard PCR with the respective primer pairs (Fig. 1).

Real-Time PCR. All reactions were performed in the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The design of the TaqMan probes, combined with the 5′-3′nuclease activity of AmpliTaq Gold DNA polymerase (PE Applied Biosystems), allowed the direct detection of the PCR product by the release of a fluorescent reporter during the PCR. To be quantitative the measurements were performed during the exponential phase. Accordingly, reactions were characterized by the point during cycling when amplification of the PCR product was first detected, rather than the amount of PCR product after a fixed number of cycles.

PCR Conditions. cDNA (3 μl) was added to the reaction mixture, which had a total volume of 25 μl. With the TaqMan PCR core Reagent kit (PE Applied Biosystems), the concentrations used were as follows: 1× TaqMan buffer A, 5.0 mM $MgCl_2$, 0.1 mM deoxynucleotide triphosphates, 0.1 mM each of forward and reverse primer, 0.1 mM probe, and 0.025 units/μl AmpliTaq Gold DNA polymerase. The thermal conditions used were 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min. Step two and three were repeated for 50 cycles. When we used the synthesized cDNA for each tumor, the 17β-HSD types 1 and 2 and β-actin-specific sequences were amplified independently in separate reaction wells in triplicate. On the same plate we included samples for standard curves for the target genes as well as a negative control sample prepared without reverse transcriptase.

Standard Curve Method. A relative kinetic method was applied, using a standard curve. The latter was constructed with 4-fold serial dilutions of cDNA from normal human breast tissue (Clontech; Fig. 2). Standard curves were produced for the three target genes after each run. The target messages in unknown samples were quantified, using the standard curves, to determine a relative measure of the starting amount. Each sample was then normalized on the basis of its β-actin expression.

Statistical Analysis. The relationships between grouped variables were analyzed with the χ^2 test. The odds ratios comparing patients with and without recurrence for the 17β-HSD enzymes and other variables were calculated by matched logistic regression analysis.

RESULTS

We used real-time reverse transcription-PCR to investigate the mRNA expression of 17β-HSD types 1 and 2 in the tumors. All 84 tumors exhibited detectable levels of 17β-HSD type 1 with a median expression level of 1.5, using the expression level in normal breast tissue as a reference. When we divided the material by tertile values, one-third of the tumors showed expression levels ≥4 (range, 4–96), and this group was classified as having high expression. The lower and intermediate tertiles were categorized together because the corresponding tertile value was close to the normal level. 17β-HSD type 2 was detectable in 12 tumors (14%). Because of the small number of positive cases, this group was not further categorized.

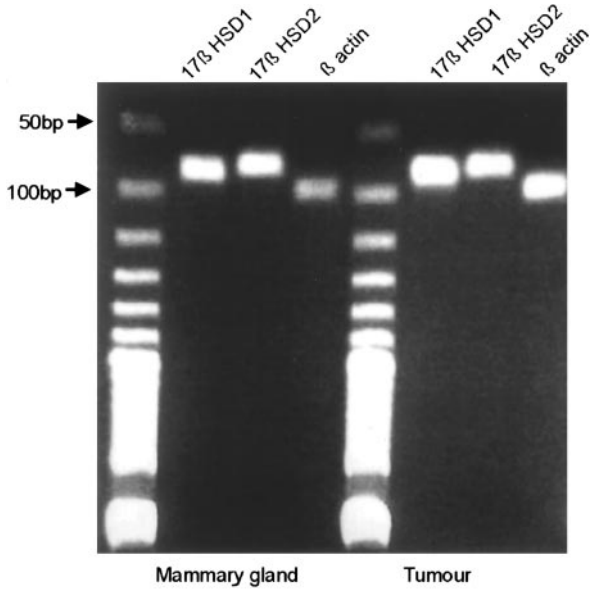

Fig. 1. cDNA products for 17β-HSD types 1 and 2 (*HSD1* and *HSD2*, respectively) and β-actin from standard PCR with the same primer pairs as those used in real-time PCR. cDNA was synthesized from total RNA from a pool of normal human breast tissue samples (*left*) and from total RNA extracted from one of the tumors expressing both types 1 and 2 (*right*).

No association was found between high expression of 17β-HSD type 1 and the status of type 2 ($P = 0.51$). The data for the enzymes in relation to other characteristics are presented in Table 2. Sixty-eight tumors were ER-positive and 16 were ER-negative. 17β-HSD type 2 was lost more frequently in ER-positive tumors compared with ER-negative tumors ($P = 0.031$). The proportion of cases with high expression of 17β-HSD type 1 tended to be greater in the group with large tumors ($P = 0.09$). Among ER-positive tumors we observed a correlation between the expression of 17β-HSD type 1 and the protein level of PgR ($r = 0.43$; $P = 0.0004$).

Results from patients with late relapse of their disease compared with patients without relapse are presented in Table 3. Patients in the recurrence group more frequently had a tumor that showed high expression of 17β-HSD type 1 ($P = 0.041$), loss of type 2 ($P = 0.050$), and DNA aneuploidy ($P = 0.0077$). Because retained expression of type 2 might be protective even if type 1 expression is high, we also analyzed a combined variable (Table 3). The same variables were significant when the analysis was restricted to ER-positive patients (Table 4). Neither PgR status nor S-phase fraction was significantly related to late relapse. In a multivariate analysis comprising the ER-positive patients, the absence of 17β-HSD type 2 combined with high expression of type 1 showed prognostic significance ($P = 0.016$) in addition to DNA aneuploidy ($P = 0.0058$), whereas PgR status did not ($P = 0.71$).

Table 1 *Primers and probes used for detection of 17β-HSD types 1 and 2*

	Forward primer	Reverse primer	Probe
17β-HSD type 1	5′-TAT GCG AGA GTC TGG CGG TT-3′	5′-TGC ACT GGG CCG CAC T-3′	5′-CGA TCA GGC TCA AGT GGA CCC CAA-3′
17β-HSD type 2	5′-TTA CCT GTG GAT CAG AAG GCA GT-3′	5′-TTG CAC AAA GCA TGG CCA-3′	5′-CCC GCA ATC ACC ACC TGT CAC CA-3′

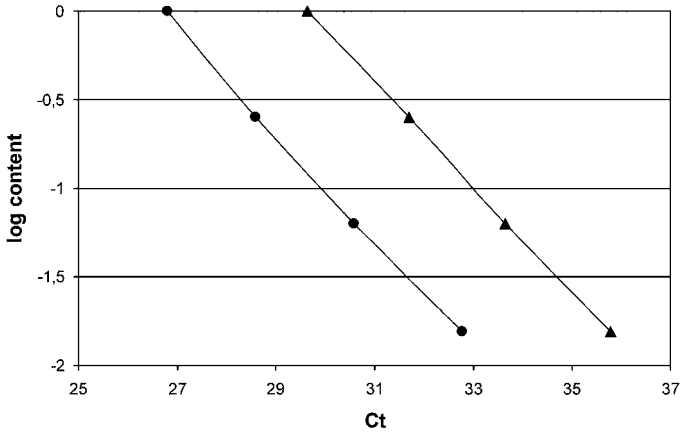

Fig. 2. Standard curves for 17β-HSD type 1 (●) and 2 (▲) constructed with 4-fold serial dilutions of cDNA from normal human breast tissue. The threshold cycle (*Ct*), the point during cycling when amplification of the PCR product is first detected, was plotted *versus* the relative content of the diluted samples (logarithmic scale). For all tumor samples, target quantities were determined from the standard curves and thus were calibrated to the quantities in normal tissue. Quantitation was normalized to β-actin (endogenous control), for which a similar standard curve was constructed (not shown).

Table 2 *Expression of 17-HSD type 1 and 2 in relation to other characteristics*

		Type 1, n (%)		Type 2, n (%)	
	n	Low	High	−	+
Nodal status					
N−	34	22 (65)	12 (35)	30 (88)	4 (12)
N+	50	34 (68)	16 (32)	42 (84)	8 (16)
Tumor size					
<20 mm	44	33 (75)	11 (25)[a]	38 (86)	6 (14)
>20 mm	40	23 (57.5)	17 (42.5)	34 (85)	6 (15)
ER status					
ER+	68	43 (63)	25 (37)	61 (90)	7 (10)[b]
ER−	16	13 (81)	3 (19)	11 (69)	5 (31)
PgR status					
PgR+	36	23 (64)	13 (36)	30 (83)	6 (17)
PgR−	48	33 (69)	15 (31)	42 (88.5)	6 (12.5)
DNA ploidy					
Diploid	27	21 (78)	6 (22)	21 (78)	6 (22)
Aneuploid	57	35 (61)	22 (39)	51 (89)	6 (11)
S-Phase fraction (n = 68)					
<10%	47	34 (72)	13 (28)	41 (87)	6 (13)
>10%	21	15 (71)	6 (29)	18 (86)	3 (14)

[a] $P = 0.09$.
[b] $P = 0.031$.

DISCUSSION

Estrogens are important mitogenic stimulators in breast cancer, and local production of estrogens is of significance in the progression of the disease. In this study we investigated the expression of 17β-HSD types 1 and 2 in breast carcinomas and found that abnormal expression of the enzymes was related to late events of distant metastasis. Speirs *et al.* (11) previously showed that the reductive pathway (E1→E2) is the dominant in malignant breast tumors, whereas the oxidative activity (E2→E1) is higher in the normal breast. The same group and others have also shown that 17β-HSD types 1 and 2 play a role in the local estrogen metabolism, especially in postmenopausal women, after ovarian estrogen production has ceased (9, 10).

The 17β-HSD types 1 and 2 proteins have been investigated with immunohistochemistry, and in a recent study 17β-HSD type 1 was detected in 61% of the ductal carcinomas, whereas type 2 was not detected in all cases examined (7). The authors suggested that 17β-HSD type 1 is the principal enzyme in breast tumors (7), and a recent study found that a polymorphism in the gene for 17β-HSD type 1 can be used to identify women at increased risk for advanced breast cancer (12). We found detectable expression levels of 17β-HSD type 1 in all of the tumors, whereas many tumors lacked expression of type 2, in particular ER-positive tumors. On the other hand, type 2 was expressed in all of the samples from normal mammary gland and in 31% of the ER-negative tumors. The loss of 17β-HSD type 2 activity could result in a significant increase of the more biologically active E2, and it might be an important mechanism in the pathogenesis of ER-positive breast neoplasm. The question is: how is type 2 lost in most of these tumors, whereas it is expressed in the normal mammary gland? Some authors have suggested that different kinds of cytokines, particularly interleukin 6 and 8, can act as cofactors that can regulate the expression of 17β-HSD (10, 13). The local steroid hormones may also up- or down-regulate the enzyme; it is known that progesterone up-regulates type 2 in the human endometrium (14). It is also known from earlier studies that the expression of 17β-HSD varies during the menstrual cycle (15). There was no evident negative correlation in the present study between the expression of the type 1 and 2 enzymes, which may indicate that the loss of type 2 expression and the overexpression of type 1 is not simply a question of gene regulation caused by estrogen. Hypermethylation or allelic loss of the gene locus may be alternative causes of loss of expression. The gene encoding 17β-HSD type 2 is located at 16q24.1-2, and previous studies have shown that LOH at 16q is more frequent in ER-positive tumors (16, 17). LOH at 16q is an early and frequent event in breast cancer (16–20) and has been identified as an independent marker of good prognosis (21). In contrast, a study of familial breast carcinomas, not including patients with early recurrence (because the patients were selected from all living patients from two clinics), indicated that LOH at 16q was associated with increased risk of distant metastasis (22). Similarly, we found that loss of 17β-HSD type 2 expression was correlated with late relapse. The fact that expression of type 2 was found more often in the ER-negative tumors would rather support that the presence of type 2 indicates poor prognosis. However, this might be the case with short-term follow-up when patients with early recurrence are not excluded because of the study design. In prostate cancer, LOH at 16q24.1-24.2 is a common event and is associated with metastatic and aggressive behavior (23, 24). Among several genes in the region that may be involved in prostate carcinogenesis, the authors discuss whether the activity of 17β-HSD type 2 protects prostatic epithelial cells from excessive androgen action and reduces the proliferative pressure on

Table 3 *Odds ratios for patients with or without relapse in relation to 17-HSD isoforms and other variables*

	Patients with relapse (*n*)	Patients without relapse (*n*)	Odds ratio[a] (95% CI[b])	*P*
17-HSD type 1				
Low	24	32	1.0	0.041
High	18	10	3.0 (1.0–12.6)	
17-HSD type 2				
−	39	33	1.0	0.050
+	3	9	0.25 (0.05–1.2)	
17-HSD type 1/2				
Low/± or high/+	25	34	1.0	0.016
High/−	17	8	4.0 (1.1–14.2)	
ER status				
ER+	36	32	1.0	0.20
ER−	6	10	0.44 (0.12–1.7)	
PgR status				
PgR+	17	19	1.0	0.65
PgR−	25	23	1.2 (0.5–2.9)	
DNA ploidy				
Diploid	7	20	1.0	0.0077
Aneuploid	35	22	3.2 (1.3–7.9)	
S-Phase fraction[c]				
<10%	18	21	1.0	0.36
>10%	9	6	1.8 (0.5–6.0)	

[a] Matched logistic regression.
[b] CI, confidence interval.
[c] Data available for 54 patients.

Table 4 *Odds ratios for ER-positive patients with or without relapse in relation to 17-HSD isoforms and other variables*

	Patients with relapse (*n*)	Patients without relapse (*n*)	Odds ratio[a] (95% CI[b])	*P*
17-HSD type 1				
Low	15	22	1.0	0.028
High	14	7	4.5 (1.0–20.8)	
17-HSD type 2				
−	29	24	1.0	0.025
+	0	5	0 (0–1.1)	
17-HSD type 1/2				
Low/± or high/+	15	23	1.0	0.016
High/−	14	6	5.0 (1.1–22.8)	
PgR status				
PgR+	13	14	1.0	0.80
PgR−	16	15	1.1 (0.42–3.1)	
DNA ploidy				
Diploid	4	15	1.0	0.0055
Aneuploid	25	14	4.7 (1.3–16.2)	
S-Phase fraction[c]				
<10%	13	16	1.0	0.15
>10%	5	2	3.0 (0.6–15.0)	

[a] Matched logistic regression.
[b] CI, confidence interval.
[c] Data available for 36 patients.

prostatic cells (23, 24). Moreover, analysis of 17β-HSD activity in colonic mucosa indicated that the predominant activity was oxidative (E2→E1) and that this conversion was significantly lower in colon tumors compared with normal mucosa, suggesting that loss of estrogen inactivation may be a mechanism in the pathogenesis of colonic cancer (25, 26).

A correlation between 17β-HSD type 1 expression and ER status did not reach statistical significance in the present study. However, when PgR content and 17β-HSD type 1 were analyzed as continuous variables, a significant correlation was found in ER-positive tumors ($r = 0.43$; $P = 0.0004$). Because the PgR gene is a target gene for activated ER, this correlation supports the view that high expression of 17β-HSD type 1 gives rise to increased action of estrogen.

A high level of 17β-HSD type 1 expression or no expression of type 2 indicated increased risk of late relapse in breast cancer in the present study. A late relapse may be related to the natural course of the tumor, but it might also be the result after completion of an effective treatment. Long-term adjuvant tamoxifen is beneficial compared with treatment of shorter duration (27). Therefore, the 17β-HSD enzymes may be potential predictors of patients who could benefit from long-term treatment with tamoxifen or inhibitors of enzymes involved in estrogen synthesis. Some ER-positive patients with metastatic disease who have failed on tamoxifen treatment still show response to aromatase inhibitors (28). This indicates that tamoxifen does not fully block the action of estrogen in some patients, possibly because of high levels of E2. If this holds true, overexpression of 17β-HSD type 1 might be a cause of tamoxifen resistance.

To the best of our knowledge, this is the first study to show that abnormal expression of 17β-HSD types 1 and 2 may have prognostic implications in breast cancer. Although breast tumors have shown to be negative for type 2 in immunohistochemical studies, we found that some cases still expressed type 2, in particular ER-negative tumors. The importance of these findings needs to be studied further, and it remains to be proven that altered expression at the RNA level translates to abnormal protein expression.

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