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Expression of progesterone metabolizing enzyme genes (AKR1C1, AKR1C2, AKR1C3, SRD5A1, SRD5A2) is altered in human breast carcinoma

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Abstract

Background: Recent evidence suggests that progesterone metabolites play important roles in regulating breast cancer. Previous studies have shown that tumorous tissues have higher 5 α -reductase (5 α R) and lower 3 α -hydroxysteroid oxidoreductase (3 α -HSO) and 20 α -HSO activities. The resulting higher levels of 5 α -reduced progesterone metabolites such as 5 α -pregnane-3,20-dione (5 α P) in tumorous tissue promote cell proliferation and detachment, whereas the 4-pregnene metabolites, 4-pregnen-3 α -ol-20-one (3 α HP) and 4-pregnen-20 α -ol-3-one (20 α DHP), more prominent in normal tissue, have the opposite (anti-cancer-like) effects. The aim of this study was to determine if the differences in enzyme activities between tumorous and nontumorous breast tissues are associated with differences in progesterone metabolizing enzyme gene expression.

Methods: Semi-quantitative RT-PCR was used to compare relative expression (as a ratio of 18S rRNA) of 5 α R type 1 (SRD5A1), 5 α R type 2 (SRD5A2), 3 α -HSO type 2 (AKR1C3), 3 α -HSO type 3 (AKR1C2) and 20 α -HSO (AKR1C1) mRNAs in paired (tumorous and nontumorous) breast tissues from 11 patients, and unpaired tumor tissues from 17 patients and normal tissues from 10 reduction mammoplasty samples.

Results: Expression of 5 α R1 and 5 α R2 in 11/11 patients was higher (mean of 4.9- and 3.5-fold, respectively; $p < 0.001$) in the tumor as compared to the paired normal tissues. Conversely, expression of 3 α -HSO2, 3 α -HSO3 and 20 α -HSO was higher (2.8-, 3.9- and 4.4-fold, respectively; $p < 0.001$) in normal than in tumor sample. The mean tumor:normal expression ratios for 5 α R1 and 5 α R2 were about 35–85-fold higher than the tumor:normal expression ratios for the HSOs. Similarly, in the unmatched samples, the tumor:normal ratios for 5 α R were significantly higher than the ratios for the HSOs.

Conclusions: The study shows changes in progesterone metabolizing enzyme gene expression in human breast carcinoma. Expression of SRD5A1 (5 α R1) and SRD5A2 (5 α R2) is elevated, and expression of AKR1C1 (20 α -HSO), AKR1C2 (3 α -HSO3) and AKR1C3 (3 α -HSO2) is reduced in tumorous as compared to normal breast tissue. The changes in progesterone metabolizing enzyme expression levels help to explain the increases in mitogen/metastasis inducing 5 α P and decreases in mitogen/metastasis inhibiting 3 α HP progesterone metabolites found in breast tumor tissues. Understanding what causes these changes in expression could help in designing protocols to prevent or reverse the changes in progesterone metabolism associated with breast cancer.

Background

In vitro metabolism studies have shown that mammary tissue from mouse [1,2], rat [3-5], cat [6], dog [6,7] and human [8-10] can convert progesterone to metabolites whose formation requires the action of the enzymes, 5 α -reductase (5 α R), 3 α -hydroxysteroid oxidoreductase (3 α -HSO) and 20 α -HSO. Differences between normal and tumor mammary gland tissues in the progesterone metabolizing enzyme (PME) activities have been noted [2,3,8-10]. Our recent observations indicate that 5 α -reduced metabolites (5 α -pregnanes) are produced at a significantly higher rate in tumorous than in nontumorous human breast tissue, indicating increased 5 α R activity in the carcinoma. Conversely, the activities of 3 α -HSO and 20 α -HSO were higher in histologically non-tumorous breast tissues which produce more δ -4-pregnanes [10].

These observations are of particular interest because the 5 α -pregnanes and 4-pregnanes have been demonstrated to exhibit opposing effects associated with breast neoplasia. Specifically, exposure of human breast cell lines to 5 α -pregnanes results in increased proliferation and decreased attachment [10], depolymerization of F-actin [11] and decreases in adhesion plaque-associated vinculin [11]. In contrast, exposure to the 4-pregnanes results, in general, in opposite (anti-cancer-like) effects by causing suppression of cell proliferation and detachment [10,11]. Additionally, specific high-affinity receptors for the 5 α -pregnane, 5 α -pregnane-3,20-dione (5 α P) and the 4-pregnane, 3 α -hydroxy-4-pregnen-20-one (3 α HP), have been identified in the plasma membrane fractions but not in the intracellular compartments of breast cancer cells [12]. The plasma membrane-associated 5 α P and 3 α HP receptors are distinct from each other and from the classical intracellular androgen, estrogen, progesterone and corticosteroid receptors [12]. These results suggest important and distinct cancer regulating functions for endogenous progesterone metabolites and emphasize a potentially prominent role for the PMEs expressed in breast tissues.

We have recently also shown that 5 α -reductase activity is higher, whereas 3 α -HSO and 20 α -HSO activities are lower in the tumorigenic cell lines, MCF-7, MDA-MB-231, and T-47D, than in the nontumorigenic, MCF-10A, cell line [13]. The differences in enzyme activities between the tumorigenic and nontumorigenic cell lines are correlated with differences in levels of mRNA expression [13] of 5 α -reductase type 1 (5 α R1; *SRD5A1*), 20 α -HSO (*AKR1C1*), 3 α -HSO type 2 (3 α -HSO2; *AKR1C3*) and 3 α -HSO type 3 (3 α -HSO3; *AKR1C2*). A recent study [14] also noted that 3 α -HSO type 3 (*AKR1C2*) mRNA expression is lower in prostatic adenocarcinoma than in normal human prostate tissue.

Taken together, these findings suggest important auto-crine/paracrine cancer regulating functions for progesterone metabolites and suggest regulatory roles for 5 α R, 3 α -HSO and 20 α -HSO activities in human breast cancer progression. The etiology of PME activity changes during breast carcinogenesis is not known. The objective of the current studies was to determine if PME gene expression levels have been altered in breast tumorigenesis. To this end, reverse transcription (RT) polymerase chain reaction (PCR) was used to quantify expression levels of 5 α R1, 5 α R2, 20 α -HSO, 3 α -HSO2 and 3 α -HSO3 in paired (tumorous and nontumorous) tissues from 11 patients, and unpaired tumor tissues from 17 patients and nontumorous breast tissues from 10 reduction mammoplasty samples. The results provide the first evidence that expression levels of *SRD5A1/2* are elevated whereas those of *AKR1-3* are markedly decreased in breast carcinoma. The findings suggest that selective changes in specific PME expression levels could lead to local increases in carcinoma-promoting 5 α P and decreases in carcinoma-inhibiting 3 α HP and 20 α DHP in breast cancer tissue.

Methods

Breast tissues

Tissues were obtained from Tissue and Archives Committee, Department of Pathology, London Health Sciences Centre, London, Ontario. They were previously collected (1995-1999) tissues that had been stored in liquid nitrogen freezers. The tissues were provided after being anonymized and their use for these studies was approved by the Interagency Advisory Panel on Research Ethics and the local Institutional Ethics Review Board. The tissues are numbered arbitrarily for convenience in these investigations. Paired samples were obtained from 11 patients and in each case consisted of a tissue sample from the tumor biopsy and a sample of tissue away from the tumor (nontumor tissue). Histological examination of the grossly normal breast tissue confirmed the absence of carcinoma or significant lymphocyte infiltration. Pathologists graded the tumors by the Scarff-Bloom-Richardson (SBR) histological grading system as part of their routine evaluation. The degree of lymphocytic infiltration was evaluated separately using a semi-quantitative method. Estrogen (ER) and progesterone (PR) receptors were assayed in cytosols prepared from quick frozen tissues using quantitative enzyme immunoassay kits purchased from Abbott Laboratories (Abbott Park, IL). Table 1 gives details of patients and tumor tissues of the paired samples. In addition, 17 tumor samples (without matching nontumor tissue) and 10 samples from reduction mammoplasty specimens were also examined for enzyme messenger RNA (mRNA) expression levels.

Table 1: Patient and biopsy information from the tumors of paired (tumor and normal from the same breast) tissue samples.

Patient No.	Diagnosis ^a	SBR Grade ^b	Age	ER ^c	PR ^c	Malignant lymph nodes ^d
1	IDC	III/III	43	-	-	NS
2	IDC	III/III	55	-	-	0/11
3	IDC	III/III	67	-	-	0/17
4	IDC	III/III	49	-	-	NS
5	IDC	III/III	59	+++	+++	0/9
6	IDC	III/III	75	-	-	0/10
7	IDC	II/III	60	-	-	15/15
8	IDC	III/III	34	-	-	1/15
9	IDC	III/III	70	++++	-	0/5
10	IDC	II/III	69	na	na	0/16
11	IDC	III/III	49	++	+	0/8

^a IDC: infiltrating duct carcinoma ^b SBR: Scarff-Bloom-Richardson histological grading system ^c ER and PR concentration codes: - receptor values less than 24 + receptor values between 25 and 99 ++ receptor values between 100 and 249 +++ receptor values between 250 and 549 ++++ receptor values > 550 ^d Number of lymph nodes assessed as malignant out of total number examined na: not available NS: No lymph nodes submitted with specimen

RNA isolation and reverse transcription

RNA isolation and reverse transcription (RT) was as described [13]. Briefly, the procedure was as follows. Total RNA was isolated from the tissues by homogenization in TRIzol[®] reagent (Invitrogen) and extraction following the manufacturer's instructions. Prior to reverse transcription, RNA was treated with DNase I for 30 minutes at 37°C using a DNA-free[™] kit (Ambion). Complementary DNA (cDNA) was obtained from 2.0 µg of DNase treated total RNA using 200 U Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen) and random hexamer primers (Invitrogen) in a total volume of 20 µl following the manufacturers instructions. To exclude any amplification of genomic DNA, all experiments included conditions in which the reverse transcriptase enzyme was omitted. In addition, no template (NT) controls were run for both the RT and PCR stages for each of the primer sets and none showed any visible PCR product.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

All PCR primers were purchased from Invitrogen Canada (Burlington, ON) and primer sequences, PCR conditions, PCR reaction kinetics and quantification were determined as previously described [13]. The conditions were as follows: 95°C denature for 4 minutes followed by cycling, each cycle with a 20 second denature at 94°C, a 30 second anneal at 62°C, and a 30 second extension at 72°C. Cycling was followed by a final extension for 4 minutes at 72°C. For each reaction an aliquot of cDNA was amplified in a 25 µl total volume using 1.25 U of Platinum Taq DNA Polymerase (Invitrogen), 2 mM MgCl₂, 0.2 mM dNTP's, and 2 mM primer. Samples were amplified in separate reactions for 12 cycles with the 18S ribosomal RNA (rRNA) primers, 27 cycles with the 5αR1, 20α-HSD, 3α-

HSD1, 3α-HSD2 and 3α-HSD3 primers, and 33 cycles with the 5αR2 primers. Prior studies had shown that under these cycling conditions, quantification was linear over an 8-fold range [13]. The PCR products were separated on 9% polyacrylamide gels and visualized using ethidium bromide staining. The bands were quantified by Quantity One 4.2.1 Gel Doc Software (BioRad Laboratories). The quantity of each band is expressed as total pixel value, based on intensity and number of pixels per band. Results are given as ratios of the total pixel value of the band of interest to the total pixel value of the 18S band. Values were obtained from the average of duplicate PCRs run on the same gel and assays were repeated three times for each gene, using a fresh cDNA sample each time.

Statistics

Results are given as mean ± SEM. Data were analyzed either by Student's t test for two columns or by ANOVA followed by Student-Newman-Keuls test comparing all columns. Differences were considered significant if p < 0.05.

Results

Paired tissues

Table 1 summarizes information on the eleven patients and the tumor portion from each paired tissue. The ages of the women at the time of surgery ranged from 34 to 75 years. The tumorous tissues were diagnosed as infiltrating ductal carcinomas of no special type. They were graded by the Scarff-Bloom-Richardson (SBR) histological grading system, whereby two carcinomas were Grade II/III and nine were III/III. Estrogen (ER) and progesterone (PR) receptor information was available for 10 of the tumor tissues. Eight tumor tissues were ER- and PR-negative, two were ER- and PR-positive, and one was ER-positive and

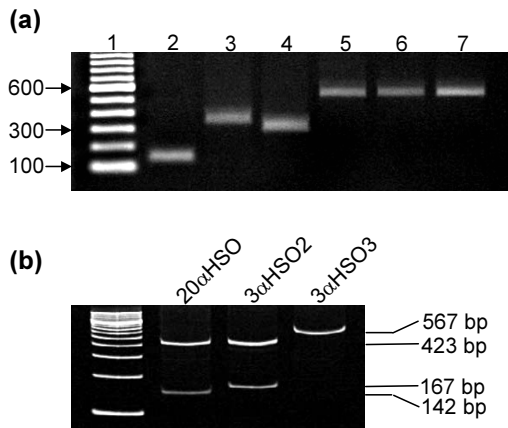


Figure 1
 Visualization of reverse transcribed (RT) mRNA from human breast tissue that was PCR amplified with primers specific for progesterone metabolizing enzyme genes. (a) The PCR products were initially identified by separation on a 1.5% agarose gel. The products and number of cycles are as follows: Lane 2, 18S rRNA (12 cycles), Lane 3, 5αR1 (26 cycles), Lane 4, 5αR2 (33 cycles), Lane 5, 20α-HSO (27 cycles), Lane 6, 3α-HSO2 (27 cycles), and Lane 7, 3α-HSO3 (27 cycles). (b) Restriction of the HSOs with *PvuII* and *BamHI* resulted in different fragments for 20α-HSO (lane 2; 423 bp, 142 bp), for 3α-HSO2 (lane 3; 423 bp and 167 bp) and for 3α-HSO3 (lane 4; 567 bp) when separated on a 9% polyacrylamide gel. The 26 bp fragments for 20α-HSO and 3α-HSO3 are not shown on the gel image.

PR-negative. ER and PR concentrations were graded as indicated in Table 1. Two of the patients had axillary lymph nodes that contained metastases. On pathological examination, tumor was found to occupy from 25% – 75% of the frozen tissue. Normal breast parenchyma occupied from 5% – 15% of the normal breast sample with the bulk of the tissue composed of fibrous tissue and fat. Lymphocyte infiltration ranged from mild to heavy in the tumor samples and was essentially absent in the normal (nontumorous) tissues.

Expression levels of progesterone metabolizing enzymes in paired tissues

Figure 1 shows that mRNA expression of 5αR1 (SRD5A1) mRNA, 5αR2 (SRD5A2) mRNA and 18S rRNA were detected as single bands (368 bp for 5αR1; 315 bp for 5αR2 and 131 bp for 18S) by our RT-PCR protocol. Product identities were confirmed by agarose gel electrophore-

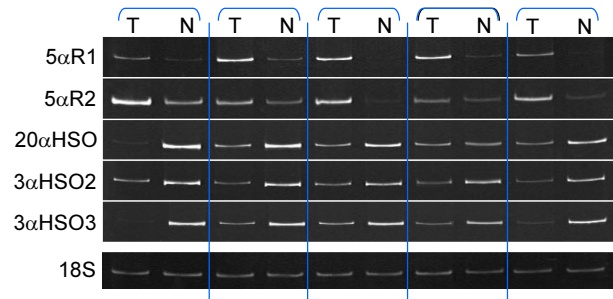


Figure 2
 Representative RT-PCR results (gels) for 5 of the 11 tumor and paired normal breast tissue samples. For each tissue, 2 µg of total RNA was reverse transcribed using random primers, and aliquots of cDNA were PCR amplified, as described in the methods, with each of 6 primer sets. For each tissue, separate cDNA samples were amplified with primers specific to 18S rRNA (12 cycles), 5αR1 primers (27 cycles), 5αR2 (33 cycles), 20α-HSO, 3α-HSO2 and 3α-HSO3 (27 cycles each). Products were separated on 9% polyacrylamide gels. Note that intensity and abundance of 5αR1 and 5αR2 bands is greater, whereas that of the HSO bands is less in tumor than paired normal tissue samples.

sis and PCR product sequencing (Robarts Research Institute sequencing facility, London, Ontario). Figure 1 also shows detection of 20α-HSO (*AKR1C1*), 3α-HSO type 3 (3α-HSO3; *AKR1C2*) and 3α-HSO type 2 (3α-HSO2; *AKR1C3*) mRNA's as single bands (591 bp for *AKR1C1*, and 590 bp for *AKR1C2* and *AKR1C3*). AKR product identities were confirmed by agarose gel electrophoresis, PCR product sequencing and double restriction enzyme digestion with *PvuII* and *BAMHI*. Restriction digestion with these enzymes produces three fragments for *AKR1C1* (423 bp, 142 bp and 26 bp), two fragments for *AKR1C2* (567 bp and 23 bp) and two fragments for *AKR1C3* (423 bp and 167 bp) as shown in Figure 1b.

Figure 2 shows examples of RT-PCR analyses of matched tumorous and nontumorous tissues from five patients for mRNA expression of 5αR1, 5αR2, 20α-HSO, 3α-HSO2 and 3α-HSO3 in relation to 18S rRNA expression. The images show that 5αR1 and 5αR2 mRNA expression was higher in tumor tissue than in nontumor tissue, whereas expression of 20α-HSO, 3α-HSO3 and 3α-HSO2 mRNAs was higher in nontumorous (normal) than in tumor tissue.

Quantitative analyses of the enzyme mRNA expressions (as ratios against respective 18S rRNA) from the paired tissues (tumor and nontumor [normal]) from the 11

Table 2: Expression levels of progesterone metabolizing enzymes (standardized against 18S mRNA) in tumorous and non-tumorous (Normal) resected breast tissues from 11 patients (described in Table 1).

Patient No.	5 α R1 (SRD5A1) ^a		5 α R2 (SRD5A2) ^b		20 α -HSO (AKR1C1) ^a		3 α -HSO-3 (AKR1C2) ^c		3 α -HSO-2 (AKR1C3) ^a	
	Normal	Tumor	Normal	Tumor	Normal	Tumor	Normal	Tumor	Normal	Tumor
1	1.68 ± 0.06	5.51 ± 0.30	0.99 ± 0.02	1.82 ± 0.10	0.76 ± 0.05	0.79 ± 0.11	0.16 ± 0.02	0.1 ± 0.01 ^d	1.25 ± 0.05	1.11 ± 0.09
2	0.20 ± 0.01	3.42 ± 0.44	0.11 ± 0.02	3.49 ± 0.03	3.77 ± 0.26	0.31 ± 0.03	3.24 ± 0.09	0.18 ± 0.02	3.81 ± 0.13	0.49 ± 0.02
3	0.64 ± 0.06	1.26 ± 0.08	0.48 ± 0.05	1.79 ± 0.09	4.31 ± 0.28	1.12 ± 0.05	3.78 ± 0.33	0.89 ± 0.05	4.01 ± 0.03	1.12 ± 0.20
4	0.88 ± 0.12	2.68 ± 0.12	0.28 ± 0.05	0.20 ± 0.02	7.41 ± 0.25	0.42 ± 0.02	7.20 ± 1.09	0.12 ± 0.01	7.77 ± 0.93	0.65 ± 0.07
5	0.38 ± 0.02	1.44 ± 0.10	0.92 ± 0.16	4.06 ± 0.33	6.07 ± 0.40	0.19 ± 0.03	6.94 ± 0.41	0.17 ± 0.02	6.54 ± 0.28	2.99 ± 0.17
6	0.66 ± 0.02	5.08 ± 0.12	0.94 ± 0.05	1.91 ± 0.15	6.04 ± 1.07	2.42 ± 0.07	5.75 ± 0.98	2.18 ± 0.10	8.31 ± 0.48	1.97 ± 0.09
7	0.20 ± 0.01	4.40 ± 0.46	0.26 ± 0.02	2.28 ± 0.12	4.79 ± 0.60	1.78 ± 0.06	6.01 ± 1.09	2.39 ± 0.03	6.07 ± 0.27	2.52 ± 0.15
8	0.58 ± 0.08	4.70 ± 0.12	0.65 ± 0.10	0.77 ± 0.02	1.51 ± 0.05	1.71 ± 0.13	1.89 ± 0.06	5.26 ± 0.88	2.83 ± 0.35	6.05 ± 0.42
9	0.20 ± 0.01	2.58 ± 0.12	0.38 ± 0.11	2.41 ± 0.24	4.70 ± 0.83	1.50 ± 0.16	5.59 ± 0.25	0.67 ± 0.14	6.63 ± 0.54	1.47 ± 0.10
10	1.08 ± 0.28	4.32 ± 0.22	0.61 ± 0.09	2.34 ± 0.14	5.50 ± 0.51	0.68 ± 0.09	4.66 ± 0.42	0.72 ± 0.14	5.25 ± 0.11	1.52 ± 0.14
11	1.32 ± 0.18	3.08 ± 0.26	1.22 ± 0.15	2.46 ± 0.09	6.04 ± 0.95	0.56 ± 0.07	6.32 ± 1.01	0.42 ± 0.07	5.78 ± 0.10	1.04 ± 0.08
Mean (±SEM)	0.709 (0.147)	3.496 (0.43)	0.614 (0.105)	2.139 (0.326)	4.627 (0.604)	1.044 (0.217)	4.685 (0.667)	1.191 (0.472)	5.296 (0.644)	1.905 (0.473)

Values were obtained from the average of duplicate PCRs run on the same gel and assays were repeated three times for each gene, using a fresh cDNA sample each time. Values are presented as mean ± SEM, n = 3. ^a Values were obtained by dividing the calculated intensity of 5 α R1 or HSO bands (27 cycles) by the calculated intensity of the 18S band (12 cycles). ^b Values were obtained by dividing the calculated intensity of 5 α R2 bands (33 cycles) by the calculated intensity of the 18S band (12 cycles). ^c Values obtained from very faint bands, calculated as <0.1, were adjusted to 0.1.

patients are shown in Table 2. Tissues from 11/11 patients showed higher levels of 5 α R1 (SRD5A1) expression in tumor than in the normal tissue. The mean (±SEM) expression value of 5 α R1 for the tumorous tissues (3.496 ± 0.43) was significantly higher (p < 0.0001) than for the paired nontumorous tissues (0.709 ± 0.147). Tissues from 10/11 patients showed higher levels of 5 α R2 (SRD5A2) expression in tumor than in the normal tissue. The mean (±SEM) expression value of 5 α R2 for the 11 tumorous tissues (2.139 ± 0.326) was significantly higher (p = 0.0002) than for the paired nontumorous tissues (0.614 ± 0.105). Tissues from 9/11 patients showed lower levels of 20 α -HSO (AKR1C1) expression in tumor than in the normal tissue. The mean (±SEM) expression value of 20 α -HSO for the 11 tumorous tissues (1.044 ± 0.217) was significantly lower (p = 0.0001) than for the paired normal tissues (4.627 ± 0.604). Tissues from 10/11 patients showed lower levels of 3 α -HSO3 (AKR1C2) expression in tumorous than in the paired nontumorous tissue. The mean (±SEM) expression value of 3 α -HSO3 for the 11 tumorous tissues (1.191 ± 0.472) was significantly lower (p = 0.0005) than for the paired nontumorous tissues (4.685 ± 0.667). Tissues from 10/11 patients showed lower levels of 3 α -HSO2 (AKR1C3) expression in tumorous than in the paired nontumorous tissue. The mean (±SEM) expression value of 3 α -HSO2 for the 11 tumorous tissues (1.905 ± 0.473) was significantly lower (p < 0.0001) than for the paired nontumorous tissues (5.296 ± 0.644).

The changes in progesterone metabolizing enzyme expression levels due to carcinoma are shown (Figure 3) by the Tumor:Normal (T/N) ratios calculated for each of the matched tissues. The mean T/N expression ratios (±SEM) for 5 α R1 and 5 α R2 were 7.838 (±2.026) and 6.055 (±2.667), respectively, which were significantly higher (p < 0.01 for 5 α R1 and p < 0.05 for 5 α R2) than the T/N ratios for 20 α -HSO (0.355 ± 0.116), 3 α -HSO2 (0.48 ± 0.17) and 3 α -HSO3 (0.443 ± 0.241). Based on the overall means (shown in Fig. 3), the T/N expression of 5 α R1 was about 22-fold, 16-fold and 18-fold higher than the T/N expression of 20 α -HSO, 3 α -HSO2 and 3 α -HSO3, respectively. When calculations are based on comparisons within individual pairs of (tumor and normal) tissues, the T/N expression of 5 α R1 was 3–207-fold (mean ± SEM: 52.5 ± 18.5), 4–132-fold (mean ± SEM: 32.9 ± 11.6) and 3–308-fold (mean ± SEM: 129.6 ± 48.6) higher than the T/N expression of 20 α -HSO, 3 α -HSO2 and 3 α -HSO3, respectively (data not shown). Similarly, the T/N expression ratios for 5 α R2 were on average 33–86-fold higher than the T/N expression ratios of the HSOs.

The 5 α R1/HSO expression ratios were less than 1.0 in normal breast tissue and significantly (about 12–16-fold; p < 0.001) higher in the paired tumorous breast tissues (Figure 4). Similarly, 5 α R2/HSO expression ratios were significantly (p < 0.05) higher in tumorous than in normal tissues (data not shown).

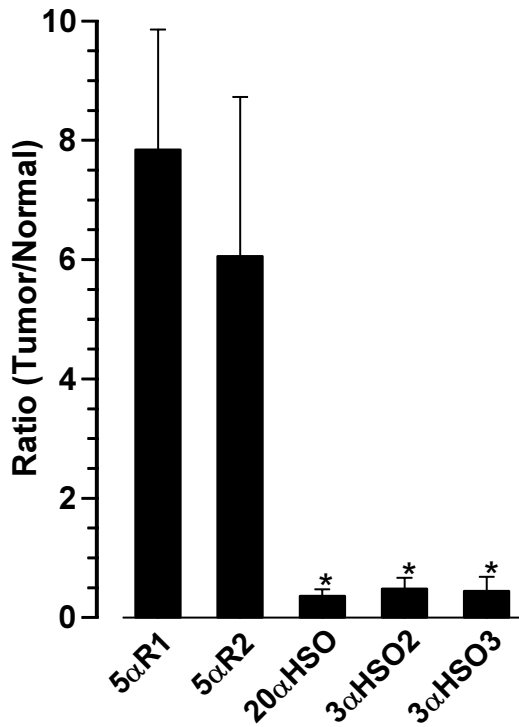


Figure 3
Gene expression levels of progesterone metabolizing enzymes in paired (tumor versus normal) human breast biopsies. Expression level of each gene (in relation to 18S rRNA) was calculated as a tumor/normal ratio for each patient. Each bar and line represents the mean ± SEM of 11 paired tissue samples. * indicates significantly different from 5αR1 at p < 0.01 and from 5αR2 at p < 0.05 (by ANOVA and Student-Newman-Keuls test).

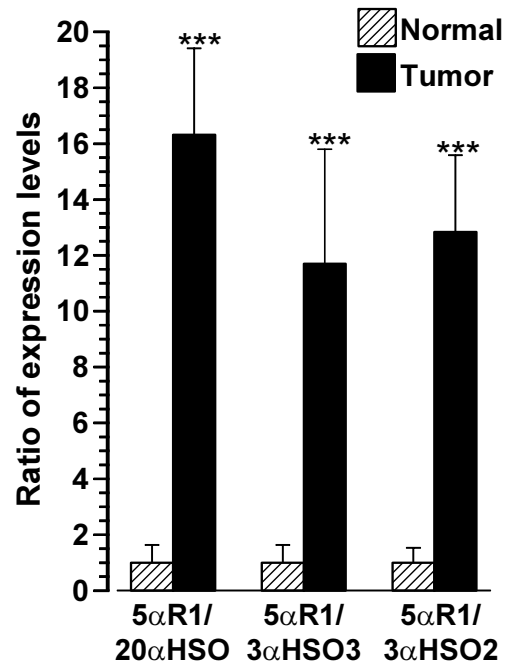


Figure 4
Expression level of 5αR1 with respect to expression level of HSD (5αR1/HSD ratio) in paired tumor and normal breast tissues. Each bar and line represents the mean ± SEM of the ratio for normal (adjusted to 1.0) and tumor. *** indicates significantly different from normal at p < 0.001, n = 11.

After the completion of the study a real-time PCR unit (Rotor Gene 3000) became available and expression levels of 5αR1 and 5αR2 (with respect to 18S rRNA) were determined on the only remaining RNA samples (from paired tissues of patients #1, #7 and #8). Expression of 5αR1 by real-time measurement, was 18.5-, 1061- and 12.5-fold higher in tumor than in normal samples from #1, #7 and #8, respectively (compared to 3.3-, 22- and 8-fold higher, respectively, by the semi-quantitative method). 5αR2 expression was higher by 6.6-, 140- and 1.3-fold by real-time measurement, (compared to 1.8-, 8.8- and 1.2-fold higher by the semi-quantitative method) in tumor than normal samples of #1, #7 and #8, respectively. The real-time results confirmed the findings by the semi-quantitative method that 5αR mRNA expression levels are higher in tumor than in normal tissue. The differences between the two methods may be due to the fact

that the real-time measurements are linear over a much wider range than the 8-fold limit of the semi-quantitative method. The real-time determinations suggest that the changes in progesterone metabolizing enzyme expression that accompany breast carcinoma may be even greater than indicated by the semi-quantitative results.

Unpaired tissues

The unpaired tissues consisted of breast carcinomas from 17 patients and normal (reduction mammoplasty) from 10 cases. The tumor tissues came from women whose ages at the time of surgery ranged from 48 to 91 years. Four of the tumor tissues were ER- and PR-negative, ten were ER-positive and PR-positive at varying receptor levels, and three were ER-positive and PR-negative. There were no evident relationships between ER/PR status and enzyme gene expression levels. Available SBR scores were Grade I for two tissues, Grade II for two tissues and Grade III for seven tissues. No apparent relationships between SBR scores and enzyme gene expression levels were detected. Of the ten samples of normal tissue three patients were

just under 20 years, five were between 24 and 35 years and two were between 47 and 53 years of age. No ER or PR data were available for these tissues.

Expression levels of progesterone metabolizing enzymes in unpaired tissues

Messenger RNA expression levels for progesterone metabolizing enzymes in tumor and reduction mammoplasty ('normal') tissues are presented in Figure 5. The calculations for expression levels of 5 α R1, 20 α -HSD, 3 α -HSD3 and 3 α -HSD2 are based on 27 PCR cycles. Expression levels of 5 α R2 were considerably lower than expression of mRNA for the other enzymes studied, requiring 34 cycles instead of 27 to visualize in the linear calculation range. The average level of expression of 5 α R1 mRNA was not significantly different between tumor and normal tissues. The average level of expression (mean \pm SEM) of 5 α R2 mRNA was more than 2-fold higher ($p < 0.01$) in tumor (1.28 ± 0.17) than in normal (0.604 ± 0.12) tissues. The average level of expression of 20 α -HSD mRNA was significantly lower ($p < 0.01$) in tumor (0.374 ± 0.048) than in normal (1.942 ± 0.691). The average level of expression of 3 α -HSD3 mRNA was significantly lower ($p < 0.01$) in tumor (0.382 ± 0.094) than in normal (1.793 ± 0.641). The average level of expression of 3 α -HSD2 mRNA was significantly lower ($p < 0.01$) in tumor (0.706 ± 0.123) than in normal (1.639 ± 0.399).

The 5 α R1 expression level was compared with each of the HSD expression levels for each tissue and the mean ratios (5 α R1/HSD) were significantly higher ($p < 0.001$ or $p < 0.01$) for tumor tissues than for normal tissues (Figure 6). The ratio of 5 α R1:20 α -HSD was greater than 1.0 in 17/17 tumor tissues (mean = 3.186; 95% CI = 2.285 and 4.086) and less than 1.0 in 6/10 normal tissues (mean = 0.867; 95% CI = 0.499 and 1.235). The ratio of 5 α R1:3 α -HSD3 was greater than 1.0 in 16/17 tumor tissues (mean = 4.732; 95% CI = 2.833 and 6.63) and less than 1.0 in 5/10 normal tissues (mean = 1.205; 95% CI = 0.432 and 1.978). The ratio of 5 α R1:3 α -HSD2 was greater than 1.0 in 12/17 tumor tissues (mean = 2.34; 95% CI = 1.433 and 3.247) and less than 1.0 in 9/10 normal tissues (mean = 0.683; 95% CI = 0.462 and 0.903). Similarly, in the non-paired tissues, 5 α R2/HSD ratios were greater than 1.0 in tumor and less than 1.0 in normal tissues and the differences were significant at $p < 0.001$ (data not shown).

Discussion

Metabolism studies have shown that mammary tissues from several species [1-6] including human [8-10] and several human breast cell lines [13,15] exhibit 5 α R, 3 α -HSD, 3 β -HSD and 20 α -HSD progesterone metabolizing activities. Differences between tumorous and nontumorous breast tissue in terms of relative activities of 5 α R, 3 α -HSD and 20 α -HSD have been observed [2,8-10]. Signifi-

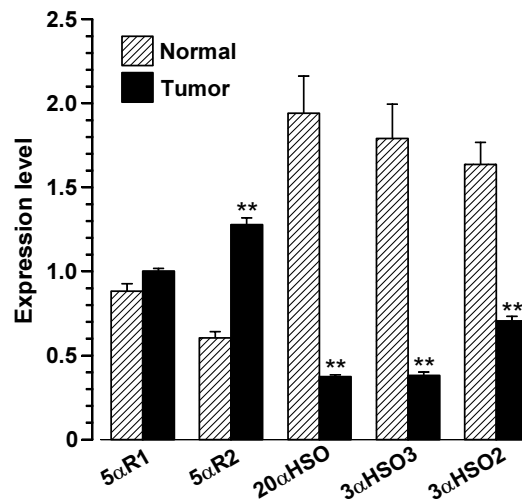


Figure 5

Gene expression of progesterone metabolizing enzymes in unpaired breast carcinoma and normal (mammoplasty) tissue samples. Expression level of each gene (in relation to 18S rRNA) from 17 tumor and 10 normal tissue samples is presented as mean \pm SEM. ** indicates significantly different from normal at $p < 0.01$.

cantly higher levels of 5 α -reduced progesterone metabolites (5 α -pregnanes) and significantly lower levels of delta-4 metabolites (4-pregnanes) are produced in tumorous than in nontumorous human breast tissue [10]. Similarly, tumor-inducing breast cell lines produce significantly higher ratios of 5 α -pregnanes:4-pregnanes than do nontumorigenic cell lines [13]. In the progesterone conversion pathway, the first 5 α -reduced metabolite is 5 α P, catalyzed by 5 α -reductase activity (Figure 7). The two 4-pregnanes resulting from direct progesterone conversion are 4-pregnen-3 α -ol-20-one (3 α HP) and 4-pregnen-20 α -ol-3-one (20 α DHP), catalyzed by the actions of 3 α -HSD and 20 α -HSD, respectively (Figure 7). The conversion to 5 α P is irreversible. The conversions to 3 α HP and 20 α DHP are reversible and depend on the relative reductive or oxidative activities of each enzyme.

The potential significance for breast cancer of the changes in progesterone metabolizing enzyme activities is that the metabolites appear to be directly involved in promoting or inhibiting tumor growth in this tissue. The 5 α -pregnane, 5 α P, which is produced at higher levels in tumorous tissues, stimulates cell proliferation and inhibits cell anchorage [10,11]. The action of the neoplasia-promoting 5 α P involves novel, specific, high-affinity receptors in the

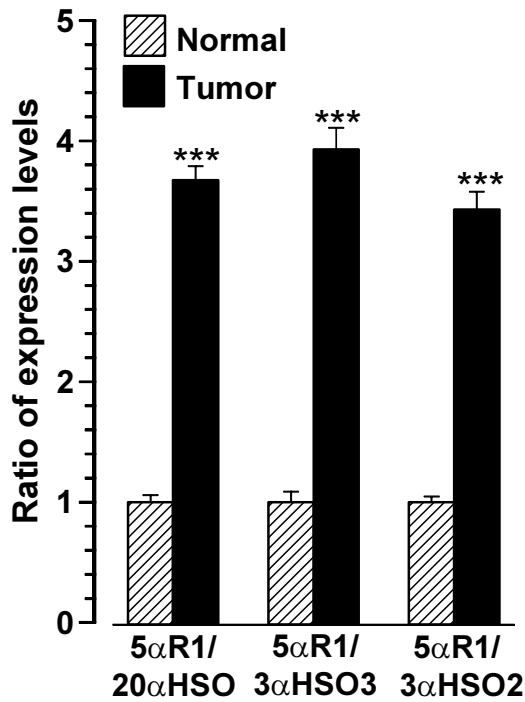


Figure 6
 Expression level of 5αR1 as a ratio of HSO expression level in unpaired tumor and normal (mammoplasty) breast tissues. Each bar and line represents the mean ± SEM of the ratio for normal (adjusted to 1.0; n = 10) and tumor (n = 17) tissues. *** indicates significantly different from normal at p < 0.001.

cell membrane [12] and results in marked alteration of the cell's cytoskeletal and adhesion complexes normally involved in cell replication and attachment [11]. On the other hand, the 4-pregnene, 3αHP, which is produced at higher levels in the normal breast tissue, has the opposite effect by suppressing cell proliferation and detachment [10,11] via separate and distinct membrane receptors [12].

Changes in enzyme activity can result from changes in the milieu in which the enzymes operate (such as temperature and pH, and concentrations of cofactors, substrates, products, competitors, ions, phospholipids and other molecules). Changes can also occur because of changes in actual enzyme amounts due to changes in the expression of the mRNA coding for the enzyme. Since the progesterone metabolizing enzyme activity studies [10] on tumorous and nontumorous breast tissue were carried out *in vitro* in identical milieus, the observed differences can be more easily ascribed to differences in enzyme amounts

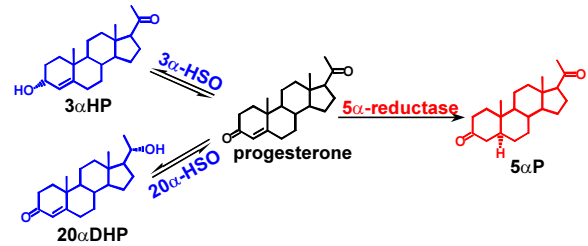


Figure 7
 Progesterone metabolizing enzyme pathways and primary metabolites affected by altered gene expression in breast carcinoma tissue. (3αHP: 4-pregnen-3α-ol-20-one; 20αDHP: 4-pregnen-20α-ol-3-one; 5αP: 5α-pregnane-3,20-dione).

resulting from altered expression. A recent study on breast cell lines has shown that differences in progesterone metabolizing enzyme activity can be correlated with changes in enzyme mRNA expression [13]. The present study indicates that changes in the mRNA expression of various progesterone metabolizing enzymes may be responsible for the previously reported [10] higher levels of 5α-pregnanes and lower levels of 3α- and/or 20α-hydroxy 4-pregnenes produced by tumorous human breast tissue.

5α-reductase expression

The enzyme responsible for the conversion of 4-ene steroids to 5α-reduced steroids is 5α-reductase (5αR; EC 1.3.99.5). Two isoforms of 5αR have been cloned and characterized in mammals, namely type 1 (5αR1) and type 2 (5αR2) [16,17]. 5αR1, which is encoded by the *SRD5A1* gene has an optimum pH of 6–9, whereas 5αR2, encoded by the *SRD5A2* gene, has an optimum pH of 5.5 [18]. 5αR1 has been detected in various androgen-independent organs, such as the liver and brain [19]. 5αR2 has been found predominantly in androgen-dependent organs, such as epididymis and prostate [17,19]. Recently 5αR1 and 5αR2 have also been located in human breast carcinoma where they were studied in relation to 5α-reduction of testosterone [20]. In the present study we observed mRNA expression for both 5αR1 and 5αR2 in all tissues examined.

The studies on the paired samples show significantly higher levels of expression of 5αR1 and 5αR2 in tumorous than in the normal (nontumorous) breast tissue biopsies (Table 2 and Fig 3). Such an increase in expression of 5αR could account for the higher levels of progesterone 5α reduction in the tumorous portion of matched breast

samples that resulted in significantly higher levels of 5 α P production [10].

In breast cell lines the higher 5 α R activity level in the tumorigenic cells [13], appeared to be attributable to 5 α R1 since its mRNA expression was several hundred fold greater than that of 5 α R2; seven more PCR cycles were required for 5 α R2 in order to show band intensity approximately equivalent to 5 α R1. Moreover, 5 α R1 mRNA expression was significantly greater, whereas 5 α R2 mRNA expression was no higher, in tumorigenic than in nontumorigenic cells [13]. Similarly, in the present studies 33 cycles were required for 5 α R2 in contrast to only 26 cycles for 5 α R1 indicating that mRNA levels for 5 α R1 greatly exceed those of 5 α R2 in human breast tissue. It was also recently demonstrated by immunohistochemistry and RT-PCR that 5 α R1 is the main isoform expressed in human breast carcinoma [20]. Together the observations provide strong evidence that 5 α R1 may be the primary 5 α -reductase expressed in breast tissue. However, in contrast to prostate where no differences were found in 5 α R2 expression between tumor and matched normal tissue [14] we saw significantly higher levels of expression of 5 α R2 in tumor than in normal breast tissue in both the matched and unmatched breast samples. Therefore it is still possible that changes in 5 α R2 mRNA expression have some relevance to progesterone metabolism in breast cancer.

In the unmatched samples the levels of 5 α R1 expression were not significantly different between normal (mostly reduction mammoplasty) and tumor tissues. Normal samples required large amounts of tissue to obtain sufficient levels of RNA for PCR analysis. Also, the unmatched tissues had been collected separately and the records do not provide information to determine if storage factors such as interval between excision and freezing may have been different. Another important factor may be the difference in age between tumor and normal samples; eight of the normal samples were derived from premenopausal women, between the ages of 19 and 35, a time when levels of steroid hormones and enzymes vary markedly during the menstrual cycle. Further investigations will be required to resolve differences in 5 α R expression at different ages and different stages of the cycle.

Hydroxysteroid oxidoreductase expression

Mammalian 3 α -HSD and 20 α -HSD activities result in stereospecific and positional keto/hydroxy interconversions. The isozymes responsible for these catalytic actions have been cloned and they belong to the AKR1C subfamily or the AKR (aldo-keto-reductase) superfamily [21]. Human 20 α -HSD is formally AKR1C1, human 3 α -HSD type 3 is formally AKR1C2 and human 3 α -HSD type 2 (identical to type 5 17 β -HSD) is formally AKR1C3. These

human isoforms share common properties and 83 to 97% sequence homology [21,22]. Despite their high homology, these isoforms display distinct differences in reactivity and substrate specificity and are expressed at different levels in different human tissues [23-26]. The expression of AKR1C1, AKR1C2 and AKR1C3 mRNAs has been detected in human mammary gland [24,27-29]. AKR1C1 has predominantly 20 α -HSD (but also some 3 α -HSD) activity, AKR1C2 has predominantly 3 α -HSD (but also some 17 β -HSD and 20 α -HSD) activity, and AKR1C3 has substantial 17 β -HSD (and lesser 3 α -HSD and 20 α -HSD) activity [14,28].

Our results show that the level of expression of these three HSD isozymes is significantly less in neoplastic than in normal (nontumorous) breast tissue from both matched and unmatched samples. On average, the level of expression of 20 α -HSD and 3 α -HSD3 is 4-5-fold lower and that of 3 α -HSD2 is about 2.5-fold lower in tumor than in normal samples. In a preliminary report in which tumorous tissue was compared to normal tissue in 24 paired cases, Stolz and co-workers, using real-time PCR, also observed that the expression of 20 α -HSD (relative to RNase P) was significantly lower (>5-fold) in the tumor portion of the majority of the cases and nearly half of these also showed decreased 3 α -HSD3 expression [30]. Their results are thus potentially similar to ours with respect to AKR1C1 and AKR1C2. Moreover, they provided evidence that the reduced AKR1C1 gene expression is associated with decreased immunohistochemical staining in tumor compared to paired normal tissue. The reduction in 3 α -HSD mRNA expression may not be confined to breast cancer. In a recent study [14] it was demonstrated that 3 α -HSD3 mRNA expression is also lower in tumor versus normal human prostate tissue samples.

The reduced expression of 3 α -(20 α)-HSD isozymes and increased expression of 5 α R isozymes in breast tumor reported here help to explain the lower and higher level of activity, respectively, of these enzymes observed in tumor versus paired normal breast tissue [10]. Similarly, tumorigenic breast cancer cell lines (MCF-7, MDA-MB-231, T-47D) also exhibited lower 3 α -HSD and 20 α -HSD and higher 5 α R expression than a non-tumorigenic (MCF-10A) cell line [13] and these differences in expression correlated with differences in respective enzyme activities [13]. Both in the tissues [10] and cell lines [unpublished], the differences in activity were reflected primarily in higher amounts of 5 α P and lower amounts of 3 α HP and 20 α DHP produced in the neoplastic versus the normal condition.

Others [14,28-31] have considered 3 α - and 20 α -reduction as catabolic pathways resulting in less active (or inactive) metabolites, thereby decreasing the amount of

biologically active ligand available for androgen and progesterone receptor binding, respectively. Thus reduced expression of *AKR1C2* in prostate cancer has been considered to be associated with reduced conversion of 5 α -dihydrotestosterone to 5 α -androstane-3 α ,17 β -diol, thereby maintaining higher levels of 5 α -dihydrotestosterone which is considered to have higher growth-promoting action [14]. Similarly, conversion of progesterone to 20 α DHP has been considered a means of decreasing the local progesterone concentrations [26]. Instead of viewing the enzymes as regulators of progesterone concentration and the progesterone metabolites as inactive by-products of this catabolism in breast tissue, we propose that the primary function of the enzymes is to regulate the relative levels of the active 5 α -pregnane and 4-pregnene progesterone metabolites. At least two of these metabolites, 3 α HP and 5 α P, have been shown to have independent specific and opposing activity with respect to breast cell proliferation, adhesion and cell cytoskeletal components [10,11]. In addition, 3 α HP and 5 α P have separate specific membrane-associated receptors on mammary cells, which are distinct from estrogen, androgen, progesterone or corticosteroid receptors [12]. Moreover, 3 α HP has been demonstrated to exhibit meiosis regulating activity in rat testis [32], inhibition of follicle-stimulating hormone release in pituitary cells [33] via nongenomic mechanisms [34-37], and analgesic [38] and anxiolytic [39] actions in the brain. It appears, therefore, appropriate to consider at least some of the progesterone metabolites produced in breast tissue as active independent hormones, potentially involved in regulating breast cancer.

5 α -Reductase and reductive 3 α -HSO and 20 α -HSO activities provide the major (if not exclusive) catabolic pathways of progesterone in breast tissues (Figure 7). The relative activities of these enzymes will determine the ratio of cancer-promoting 5 α P with respect to cancer-inhibiting 3 α HP and 20 α DHP concentrations. The current findings of the decline in expression of the HSO isozymes in tumor tissue explain the lower levels of the cancer-inhibiting steroids in relation to the cancer-promoting 5 α P. Increased expression in 5 α R mRNA would further increase the ratio in favor of cancer-promoting progesterone metabolites. We have previously suggested that the change from normal to carcinoma and/or the promotion of the carcinoma may involve an increase in the local 5 α -pregnane/4-pregnene ratio, in particular the 5 α P/3 α HP ratio. The current findings suggest that higher levels of 5 α R expression and/or lower levels of 3 α - and 20 α -HSO expression in the tumor (as compared to the normal breast tissue) result in increases in local concentrations of cancer-promoting, 5 α P, and simultaneous decreases in actively anti-neoplastic hormones such as 3 α HP. The control of the expression level of individual progesterone metabolizing enzymes might therefore provide a mecha-

nism of controlling breast cancer by controlling local concentrations of progesterone metabolites with opposite activities.

Conclusions

It is known that the progesterone metabolite, 5 α P, stimulates proliferation and detachment of breast cell lines, and thus potentially promotes mitogenesis and metastasis. The progesterone metabolites, 3 α HP and 20 α DHP, have the opposite effects by suppressing proliferation and detachment. Conversion of progesterone to 5 α P is higher, whereas conversion to 3 α HP and 20 α DHP is lower in tumor than in normal breast tissue. Progesterone conversion to 5 α P requires the action of 5 α R (SRD5A) and conversion to 3 α HP and 20 α DHP requires the actions of AKR1C1-AKR1C3 (3 α -HSO and 20 α -HSO) isozymes. The results of this study show for the first time that expression of 5 α R mRNA is significantly elevated whereas expression of AKR1C1-AKR1C3 mRNA is significantly decreased in tumor compared to normal breast tissue. The findings suggest that the shift in pattern of progesterone metabolism occurring in breast carcinoma may be due to altered expression levels of the progesterone metabolizing enzymes. Understanding what causes these changes in expression could help in designing protocols to prevent or reverse the changes in progesterone metabolism associated with breast cancer.

List of abbreviations

AKR, Aldo-Keto Reductase; 3 α -HSO, 3 α -hydroxysteroid oxidoreductase; 20 α -HSO, 20 α -hydroxysteroid oxidoreductase; 5 α R, 5 α -reductase; 5 α P, 5 α -pregnane-3,20-dione; 3 α HP, 3 α -hydroxy-4-pregnen-20-one; 20 α DHP, 20 α -dihydroprogesterone or 20 α -hydroxy-4-pregnen-3-one; RT-PCR, reverse transcription-polymerase chain reaction.

Competing interests

None declared.

Authors' contributions

JW conceived and coordinated the study, participated in its design, performed the statistical analyses and drafted the manuscript. ML carried out the RT-PCR studies and participated in drafting the manuscript. JGH provided pathological analyses and editorial comment. All authors read and approved the final manuscript.

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