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ORIGINAL PAPER

Progesterone receptors A and B and estrogen receptor alpha expression in normal breast tissue and fibroadenomas

Gisele Branchini · Lolita Schneider · Rodrigo Cericatto · Edison Capp · Ilma Simoni Brum

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Abstract Fibroadenomas are the most common benign breast tumors, occurring mainly in young women. Their responses to the hormonal environment are similar to those of normal breast tissue, which suggests that steroid receptors may play a role in tumor development. We evaluated the gene and protein expression of progesterone receptors A and B (PRA and PRB) and the protein expression of estrogen receptor α (ER-α) in fibroadenoma samples, comparing with adjacent normal breast tissue, from 11 premenopausal women. Progesterone and estradiol levels were determined. No alterations in the PRs gene and protein expression and the ER-α protein expression were observed between the follicular and luteal phases, in normal breast versus fibroadenomas. Protein levels of PRA and PRB were higher in fibroadenomas compared to normal breast tissue ($P = 0.038$ and $P = 0.031$), while the PRs mRNA levels were similar in both tissues ($P = 0.721$ and $P = 0.139$). There were no differences in ER-α protein expression between normal breast tissue and fibroadenomas ($P = 0.508$). The PRA:PRB ratio was similar in the tissues, and also showed a strong correlation in both ($r = 0.964$, $P = 0.0001$). Our data suggest a role of PRs in the growth and development of fibroadenomas, although without alterations of the PRA:PRB ratio in these tumors. The absence of alterations in ER-α protein levels could be a characteristic behavior of fibroadenomas, unlike breast cancer.

Keywords Fibroadenomas · Breast · Progesterone receptors · Estrogen receptor

G. Branchini · L. Schneider · I. S. Brum (✉)
Laboratory of Endocrine and Tumoral Molecular Biology, Department of Physiology, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500, CEP 90 050-170, Porto Alegre, RS, Brazil
e-mail: ilma@ufrgs.br

G. Branchini · L. Schneider · E. Capp · I. S. Brum
Laboratory of Molecular Gynecology and Obstetrics, Centro de Pesquisas, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil

R. Cericatto
Breast Service, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil

E. Capp
Department of Gynecology and Obstetrics, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

Introduction

Fibroadenomas (FAs) are the most common benign breast tumors [1, 2], occurring in 25% of asymptomatic women [3, 4]. These tumors affect women between 20 and 50 years of age, and are most frequent in the range of 20–30 years [2, 5]. FAs manifest as a proliferation of epithelial and stromal cells, forming a mass that usually measures 2–3 cm in the largest dimension [6].

Clonal analysis has shown that the FA consists of polyclonal epithelial and stromal cells, suggesting that it is a hyperplastic rather than a neoplastic lesion [7]. The mechanisms controlling development and growth of FAs are little understood [8]. La Rosa and co-workers [6, 9] have suggested that multiple receptor signaling pathways, including the pathways of estrogen and progesterone receptors (PRs) as well as those of growth factors and their receptors, could be involved in the growth and differentiation of benign breast lesions.

Progesterone receptors are members of the superfamily of nuclear receptors, which act as transcriptional factors that

regulate gene expression [10]. They exist naturally as two isoforms, PRA and PRB, transcribed from a single gene as a result of transcription from two alternative promoters, estrogen-induced, and also from translation initiation at two different AUG codons in the PRB mRNA [10, 11]. PRs are essential for normal breast development, and the PRA:PRB ratio has been suggested to influence the biological actions of progesterone [12, 13]. Some studies using knock-out mice for one of two PR isoforms have suggested that PRB is a stronger activator of target genes and PRA acts as a dominant repressor of PRB and other steroid receptors, indicating that PRA has the ability to diminish overall progesterone responsiveness of specific target genes in specific tissues [13–15]. Nevertheless, PRA and PRB seem to regulate different subsets of genes, and although PRB is transcriptionally more active, there are genes that are uniquely regulated by PRA [16]. A recent study showed that the effects of progesterone on gene expression are almost identical in cells with PRA predominance, compared with cells expressing nearly equivalent PRA and PRB, demonstrating that PRA does not act as a dominant inhibitor of PR activity [17].

Like progesterone, estrogens play an essential role in the reproductive tract. Estrogens act through their receptors, estrogen receptor α (ER-α) and estrogen receptor β (ER-β), which are also members of the superfamily of nuclear receptors, and induce gene expression by acting as transcription factors [18]. Unlike PRs, ER-α and ER-β are transcribed from different genes and have distinct tissue distribution as well as physiological functions. It has been suggested that ER-α is the dominant mediator of estradiol effects in reproductive tissues and the mammary gland [19, 20]. ER-α expression is closely associated with breast cancer biology, especially with tumor development [21]. We have previously demonstrated greater expression of ER-α mRNA in FAs than in normal breast tissue [22], but the expression of ER-α protein in these lesions remains to be investigated. The study of PRs and ERs in FAs may help to clarify the mechanisms of action of these steroid hormones in regulating mammary gland proliferation and differentiation. Therefore, the aims of the present study were to evaluate the gene and protein expression of PRs A and B (PRA and PRB) and the protein expression of ER-α in FA samples, compared to adjacent normal breast tissue.

Results

Of the 11 patients in the study, one was excluded because the anatomopathological diagnosis was discordant with FA (case of phyllodes tumor). The mean age of the remaining 10 participants was 25.2 years and the mean age at menarche was 13 years. The mean IMC was 21.2 kg/m^2, and the mean diameter of the FAs was 2 cm. Four patients previously had at least one full-term pregnancy.

As expected, the plasmatic levels of progesterone were different between follicular and luteal phases. One-third of the patients were using hormonal contraceptives. Progesterone levels in these patients were different from those in the luteal phase, but were not different from those in the follicular phase. We initially evaluated the PRs mRNA levels and PRs and ER-α protein levels between menstrual-cycle phases, and in the patients using a hormonal contraceptive. We also investigated correlations between gene and protein expression and progesterone and estradiol levels. No differences in gene and protein expression were observed in any of these conditions, either in normal breast tissue or in FAs (Table 1). Thus, we included all the patients in one group, and compared the results of gene and protein expression only between FAs and normal breast tissue.

We analyzed the mRNA levels of PRB and PRAB by real-time PCR. The term PRAB refers to the analysis of both PR mRNAs, PRA and PRB, through the common fragment of the two PRs isoforms, because it is not possible to assess the levels of PRA mRNA separately. Quantitative results from qPCR are shown as the ratio of the target gene versus β2-microglobulin in arbitrary units (AU). Figure 1 shows the expression of PRB and PRAB mRNA as individual values of gene expression and mean. There were no

Table 1 Protein expression of PRA, PRB, and ER-α in menstrual-cycle phases

	Normal tissue	*P* value	Fibroadenoma	*P* value
PRA				
Follicular/OC	0.0312 ± 0.007	0.359	0.0431 ± 0.005	0.309
Luteal	0.0457 ± 0.015		0.0545 ± 0.011	
PRB				
Follicular/OC	0.0349 ± 0.011	0.516	0.0425 ± 0.013	0.636
Luteal	0.0238 ± 0.010		0.0327 ± 0.013	
ER-α				
Follicular/OC	0.0394 ± 0.006	0.403	0.0440 ± 0.010	0.754
Luteal	0.0512 ± 0.002		0.0514 ± 0.019	

The values are represented as mean ± standard error of the mean (SEM)

OC Oral contraceptive

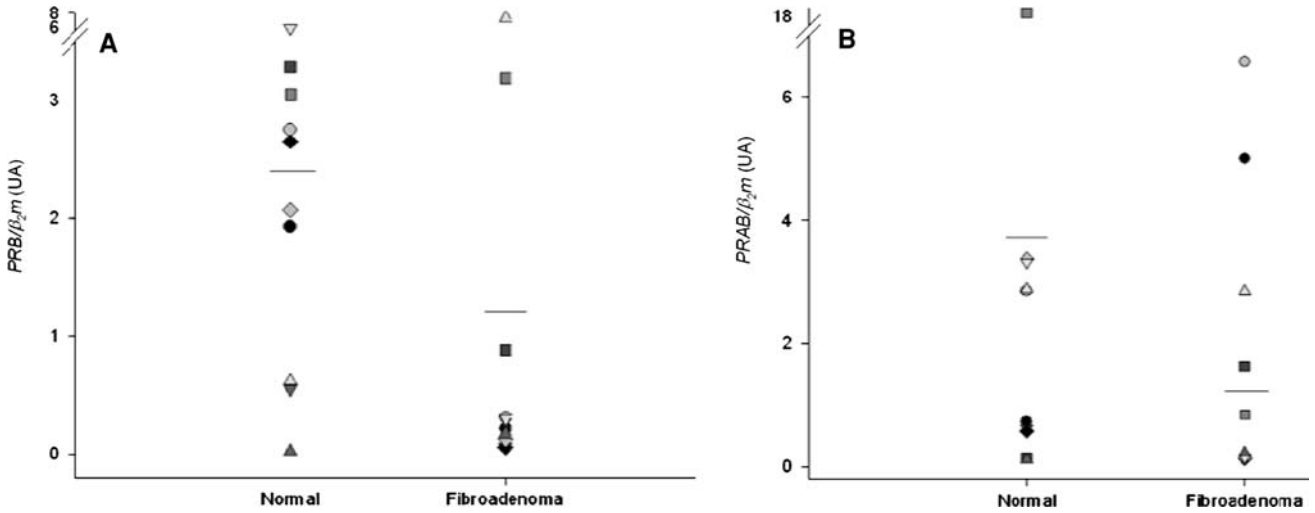

Fig. 1 PRB (**a**) and PRAB (**b**) gene expression in normal breast and fibroadenomas. Each point represents a subject and equal symbols represent the same patient. *Bars* indicate means of gene expression values. $N = 10$

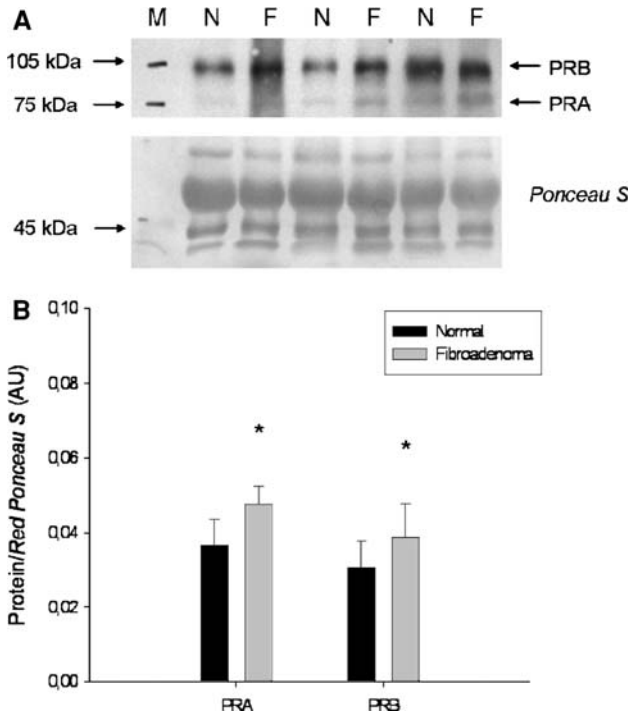

Fig. 2 PRA and PRB protein expression in normal breast and fibroadenomas. **a** Autoradiography of expression of PRA (82 kDa) and PRB (112 kDa) in three samples of normal breast (N) and fibroadenomas (F) (*upper panel*) and bands obtained by Red Ponceau S staining (*lower panel*). *M* molecular weight marker. **b** Mean ± SEM of PRA and PRB protein expression in normal breast and fibroadenomas. $N = 8$. *$P = 0.038$ and 0.031, respectively

differences in expression of PRB and PRAB mRNA between FAs and normal breast tissue ($P = 0.139$ and 0.721, respectively). PRA and PRB protein expressions are shown in Fig. 2 as mean ± SEM. PRA and PRB protein levels were higher in FAs than in normal breast tissue ($P = 0.038$ and 0.031, respectively).

The means ± SEM of ER-α protein expression in FAs and normal breast tissue are shown in Fig. 3. The ER-α expression was similar in both tissues ($P = 0.508$).

The protein expressions of PRA, PRB, and ER-α were correlated between FAs and normal breast tissue, as showed in Table 2. One negative correlation was observed between mRNA expression of PRAB and protein expression of PRA in normal breast tissue. On the other hand, one positive correlation was observed between mRNA expression of PRAB and protein expression of PRB in normal breast tissue. We also evaluated the association of gene and protein expression with clinical variables (age, nodule size, and progesterone and estradiol levels). Only age was negatively correlated with nodule diameter ($r = -0.565$, $P = 0.023$) (Table 2).

We obtained the PRA:PRB ratio of protein expression values for each patient. We did not find a statistical difference in this ratio between FAs (2.5:1) and normal breast tissue (1.6:1) ($P = 0.866$), and the PRA:PRB ratio was strongly and positively correlated between both tissues ($r = 0.964$, $P = 0.0001$).

Discussion

Progesterone receptors expression

The role of progesterone and estrogens and their receptors in the development of benign breast lesions such as FAs is uncertain, given that the main target of studies on mammary gland alterations is breast cancer. In the present study, we observed a higher expression of PRA and PRB

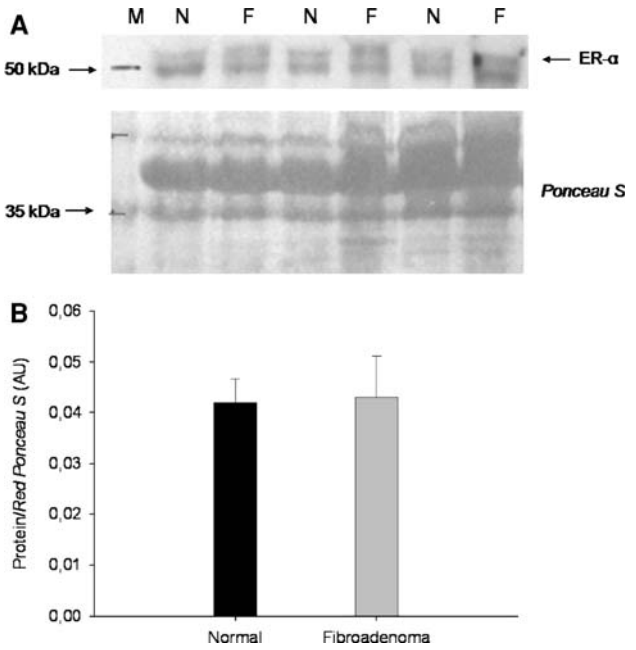

Fig. 3 ER-α protein expression in normal breast and fibroadenomas. **a** Autoradiography of expression of ER-α (66 kDa) in three samples of normal breast (N) and fibroadenomas (F) (*upper panel*) and bands obtained by Red Ponceau S staining (*lower panel*). *M* molecular weight marker. **b** Means ± SEM of ER-α expression in normal breast and fibroadenomas. $N = 10$

Table 2 Correlations of gene and protein expression in fibroadenomas and normal breast tissue

	Fibroadenoma			PRAB normal tissue
	PRA	PRB	ER-α	
Normal tissue				
PRA	$r = 0.786$	NS	NS	$r = -1$
	$P = 0.021$			$P < 0.0001$
PRB	NS	$r = 0.929$	NS	$r = 1$
		$P = 0.001$		$P < 0.0001$
ER-α	NS	NS	$r = 0.656$	NS
			$P = 0.039$	

NS Nonsignificant

proteins in FAs when compared with the matched normal circumjacent breast tissue, although the mRNA levels of PRB and PRAB were not significantly different between these tissues.

The higher levels of PRA and PRB in FAs than in normal breast tissue suggest that these proteins could be involved in cellular proliferation in such lesions. Conneely and Lydon [11] showed proliferative and differentiative effects of PRs on the development of mammary epithelium. However, the described effects of progesterone on proliferation and differentiation of the mammary gland are contradictory. Kramer et al. [23], in their study with growth factor stimulated healthy breast epithelial cells, showed a neutral effect of progesterone on the proliferation:cell death ratio. In a review of several studies, Gompel et al. [24] reported an anti-proliferative role of progesterone in normal mammary cells, either in vivo or in vitro. Other studies showed a proliferative role of progesterone in the breast [11, 13, 25, 26]. Nevertheless, this effect was mainly seen in breast cancer cells, where progesterone acts as an apoptosis inhibitor, which suggests a role of progesterone in the progression of breast cancer [25]. Notwithstanding, the cellular response to treatment with progesterone can either be proliferation, differentiation, or apoptosis, depending on the pattern of proteins present in the cell [24]. And, because PRs act in combination with multiple transcription factors, it is possible that the variability of the tissue-specific expression of the components of this multiproteic complex may result in different PRA and PRB activities in the same cell, and thus influence the cellular response to progesterone [27]. A recent work of our research group showed a modulation on p53 gene and protein expression in FAs [28]. Also, Gompel et al. [24] reported an up-regulation of p53 expression by progesterone, and a down-regulation by estrogens in normal breast cells. The induction of p53 by progesterone and the arrest of the cell cycle were also demonstrated in cultured human umbilical vein endothelial cells (HUVEC) [29]. The tumor-suppressing gene p53 acts protecting cells from malignant transformation, and the development of most tumors is associated with the loss of p53 function [30]. Thus, could be an involvement of PRs in the growth and development of FAs, however, without malignancy, through the modulation of p53 expression.

We evaluated the mRNA levels of PRs and protein levels of PRs and ER-α in the phases of the menstrual cycle, and also compared these levels with patients using hormonal contraceptives. We did not find differences in their expression, either in normal breast tissue or in FAs. Although classic studies have shown a clear regulation of hormone receptor levels by steroid hormones in different tissues, mainly in endometrial tissue [14, 31–33], in the mammary gland the levels of PRs seem not to vary between the follicular and luteal phases of the menstrual cycle [14, 32, 34]. This absence of variation could be related to a resistance to the effects of repression of receptor expression exercised by progesterone, or also to a loss of cyclicity of local hormonal flow in the mammary gland due to the presence of large amounts of adipose tissue [14, 24, 32]. Thus, we thought that there may be a progesterone-independent regulation of PR levels, possibly with involvement of these receptors, but not necessarily of progesterone, in the process of tumor development, as proposed by Jacobsen et al. [35] for breast cancer, where PRs would predict disease markers independently of progestational status. Moreover, the absence of

statistically significant differences in mRNA expression of PRs in normal tissue and tumors and the greater expression of the PR proteins in FAs suggest the existence of post-transcriptional mechanisms of regulation of PR protein expression.

The first studies of PR activity described PRB as an activator of transcription, mediating the biological effect of progesterone, and PRA as a dominant inhibitor of PRB activity and of other steroid receptors [11, 14]. However, more recent studies have shown that PRA and PRB activate different subsets of genes [16], and that PRA induces the expression of genes involved in cellular metabolism and in the regulation of cell shape and adhesion [17]. Therefore, higher levels of both isoforms of PRs seen in FAs may be involved in proliferation and differentiation of breast tissue and formation of these benign lumps, through the activation of genes directly involved in these processes.

In our study, we evaluated the PRA:PRB ratio in both tissues, and observed that this ratio did not differ between normal breast tissue and FAs. Moreover, there is a strong correlation of this proportion between normal breast tissue and FAs. The isoforms of PRs are expressed in progesterone target tissues at comparable levels, and the PRA:PRB ratio has been suggested to influence biological actions of the hormone [12]. This equivalent expression seen in normal breast cells is lost early in carcinogenesis, and predominance of one isoform, essentially PRA, is common [27, 36]. This suggests that, unlike cancers, where PRA is predominantly overexpressed, the coordinated expression of both isoforms is not lost in FAs. Furthermore, when evaluated separately, each protein showed a positive correlation of expression between the analyzed tissues.

Two studies on the PRA:PRB ratio, in which this proportion was altered to give a predominance of PRA, have indicated that the main targets of PRA are genes involved in cell shape and cellular adhesion [17, 36]. McGowan and Clarke showed that the altered PRA:PRB ratio had little effect on cell proliferation, but did have a marked effect on cell morphology. They also demonstrated that PRA can inhibit progestin action, but only when it is present in significant excess over PRB. Thus, PRA expression can fluctuate within a broad range in target cells without influencing the nature of progestin action on downstream targets, but that overexpression of PRA, as is seen in some breast cancers, may be associated with inhibition of progestin action and features of poor prognosis [36]. The predominance of PRA does not result in inhibition of progestin-mediated gene expression, and this predominance, particularly during prolonged exposure to progestin, results in acquisition of progestin responsiveness by genes involved in regulation of cellular metabolism, cell shape, and adhesion, which are normally not progestin targets [17]. The overexpression of PRA can play a role in the loss of adhesion observed in malignancy [17, 36]. Therefore, based on our observation of the strong correlation of the PRA:PRB ratio in both tissues, although this proportion was not equimolar, we can suggest a differentiated behavior of PRs in FAs, where the cells do not undergo loss of adhesion and cell shape alterations, unlike malignant cells, where PRA is overexpressed.

We observed a negative correlation between PRAB and PRA protein and a positive correlation between PRAB and PRB protein. This suggests that, within the PRA and PRB mRNAs that are analyzed together by PCR, termed PRAB, there could exist a larger amount of mRNAs encoding PRB than mRNAs encoding PRA, and hence the translation of PRA could be from translation initiation of the alternative AUG codon on PRB mRNA [10, 11].

Estrogen receptor alpha expression

In the present study, we also analyzed the protein levels of ER-α and observed similar levels of this protein in both tissues. A previous study of our research group demonstrated a higher expression of ER-α mRNA in FAs compared to normal breast tissue [22]. In contrast to gene expression, there were no differences in ER-α protein expression in both tissues. It is well established that PRs can regulate ER expression, decreasing ER levels, but this regulation seems to be cell specific [14, 33]. Our results are in agreement with this view, because we observed an increase of PR levels and unaltered levels of ER-α. Thus, PRs could be repressing a higher expression of ER-α by post-transcriptional mechanisms and in an estrogen-independent manner. It is known that, in certain circumstances, through little-understood mechanisms, PRs can be resistant to the expression repressor effect of progesterone [14, 31]. The increased levels of PRs in FAs and unaltered levels of ER-α between tumors and normal tissue found in our study may indicate that PRs may not be undergoing down-regulation by progesterone, but this hormone and its receptors could still be inhibiting ER-α expression.

Estrogen receptor α expression is closely related to breast cancer biology, especially tumor development, and it is suggested that the overexpression of this protein is a significant initial event in the genesis of mammary cancer [21]. We did not observe alterations in the expression of ER-α protein in FAs, which maintain some similarity with normal breast tissue, without the neoplastic transformations found in cancers. The positive correlation of ER-α expression observed between tissues contributes to the view that FAs behave differently from breast cancers. The entire mechanism of the pathway from ER-α leading to the proliferation and progression of mammary tumors is far from being completely clarified [21], and this also applies to PRs pathways.

To our knowledge, this study is the first to describe the higher levels of PRs in FAs, and suggests an involvement of PRs in the development of these tumors. However, ER-α seems not to be involved in the process of tumor formation, unlike breast cancer, which may be a characteristic behavior of these benign tumors. The study of gene expression alterations and/or deregulation during reproductive life and the comparison of FAs with normal tissue may help to explain hormonal influences and physiological processes involved in nodule development and evolution.

Materials and methods

Samples

The samples were obtained from premenopausal women undergoing surgical removal of breast nodules suggestive of FAs at the Breast Service, Hospital de Clínicas de Porto Alegre, Brazil. The study protocol was approved by the Institutional Ethical Committee of the Hospital de Clínicas de Porto Alegre. Eleven patients were selected for the study, and informed written consent was obtained from each subject.

The patients underwent routine preoperative assessment, with their clinical and reproductive histories recorded, as previously described [22]. Samples from the central area of the FA and from circumjacent normal mammary tissue were obtained during the surgery, identified, immediately frozen in liquid nitrogen and then transferred to a −80°C freezer for later RNA and protein extraction. The diagnosis of FA was based on the anatomopathological examination. Also during the surgery, 10 ml of venous blood was obtained in order to assess hormonal dosages.

RNA extraction

RNA was extracted from 0.2 g of each sample, by the guanidinium thiocyanate method [37] as previously described [22]. The total RNA concentration was measured by absorbance at 260 nm in a spectrophotometer for nucleic acids, and the optical density (OD) ratios (260/280 nm) were greater than 1.6.

cDNA synthesis

First-strand cDNA was synthesized from 2 μg of total RNA, using the Super Script First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies). After denaturing the RNA with Oligo(dT)12–18 primer and 10 mM dNTPmix at 65°C for 5 min, a mixture of 200 mM Tris–HCl (pH 8.4) plus 50 mM KCl, 25 mM MgCl2, 0.1 M dithiothreitol (DTT), and 40 U of RNaseOUT was added and incubated at 42°C for 2 min, followed by the addition of 50 U of reverse transcriptase and incubation at 42°C for 50 min. The mixture was heated at 70°C for 15 min to interrupt the reaction, centrifuged, and cooled on ice. About 2 U of E. coli RNase was added to the mixture and incubated for 20 min at 37°C to destroy the remaining RNA. The reaction had a final volume of 20 μl, and was stored at −20°C until used in the polymerase chain reaction (PCR).

Real-time PCR amplification

PRB and PRAB transcripts were quantified using real-time RT-PCR assays. Transcripts of β2-microglobulin were also quantified as endogenous RNA of the reference gene, to normalize each sample.

The primers to analyze β2-microglobulin mRNA were designed to span intron–exon borders, in order to prevent the amplification of any contaminating genomic DNA, and were chosen with the assistance of the computer program Primer3 [38]. The primers to analyze PRB and PRAB mRNA were obtained from the study of Sakaguchi et al. [39]. The nucleotide sequences are: β2-m sense 5′CTA TCCAGCGTACTCCAAAG3′; β2-m antisense 5′ACAA GTCTGAATGCTCCACT3′; PRB sense 5′GCCAGACCT CGGACACCTT3′; PRB antisense 5′CAGGGCCGAGGG AAGAGTAG3′; PRAB sense 5′AGAGCACTGGATGC TGTTGCT3′; PRAB antisense 5′TGGCTTAGGGCTTG GCTTT3′.

Polymerase chain reactions were performed using Power SYBR Green Master Mix (Applied Biosystems), at the DNA Engine Opticon (MJ Research, USA). Two microliters of the 5× diluted first-strand synthesis reaction were mixed with 12.5 μL of 2× Master Mix, 100 mM of 5′-end and 3′-end primers, and water to complete a final volume of 25 μL. The thermal cycling conditions comprised an initial denaturation step at 94°C for 2 min, then 40 cycles with steps depending upon the gene amplified. The PCR cycle conditions were: PRB—denaturation at 94°C for 50 s, annealing at 59°C for 40 s, and extension at 72°C for 45 s; PRAB—denaturation at 94°C for 45 s, annealing at 56°C for 30 s, and extension at 72°C for 45 s. PCR products were submitted to electrophoresis on 1.5% agarose gels in order to confirm the presence of a single band with the expected size.

A standard curve with 10 fold serial dilutions from a known amount of cDNA was used to quantify the amount of cDNA in the samples. Quantitative values were obtained from the threshold cycle (Ct) number at which the increase in fluorescent signal associated with an exponential increase of PCR products could be detected.

Western blot

To obtain tissue lysate, 0.2 g samples of FA and normal adjacent mammary tissue were individually homogenized in 500 μL of 10 mM Tris pH 7.4, 1 mM EDTA pH 7.4, 1 mM PMSF, 100 mM NaF, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4 and 1% Triton X-100. The samples were shaken overnight at 4°C and then centrifuged at 12,000 × *g* for 30 min at 4°C. Tissue lysates were applied to a 10% SDS-PAGE and transferred to a nitrocellulose membrane by electroblotting. The membrane was washed with blocking solution (NET) containing 15 mM NaCl, 5 mM EDTA, 50 mM TRIS, 0.5% Tween 20, pH 7.4 plus 2.5% BSA, and incubated with specific mouse anti-PRs (Santa Cruz Biotechnology) or mouse anti-ER-α (Santa Cruz Biotechnology) diluted in NET with 2.5% BSA and 0.1% azide. The bands were detected by a chemoluminescence reaction (ECL) with film (CL-Exposure, Pierce) exposure for 15–60 s. The OD of the bands obtained by chemoluminescence was measured by densitometric analysis with an image-processing system (ImageMaster VDS, Pharmacia Biotech). NC staining with Red Ponceau S was used to normalize the PRA, PRB, and ER-α protein amounts.

Progesterone and estradiol assays

Progesterone (P4) and estradiol (E2) were measured by electrochemiluminescent assay (Elecsys Progesterone II and Elecsys Estradiol, Roche, Switzerland), with assay sensitivities of 0.15 ng/mL and 18.4 pg/mL, respectively.

Statistical analysis

The results for PR gene expression and ER-α protein expression were analyzed as median and interquartile range. Comparison between FAs and normal tissue was performed by Wilcoxon's *T*-test. Data for PR protein expression are presented as mean ± standard error of mean (SEM). Means between groups were compared by paired Student's *t*-test. Spearman's rank correlation was used to assess the correlation of gene and protein expression in tumors and normal breast tissue, and also to analyze associations between these data and clinical variables. The level of significance adopted was $P < 0.05$. All analyses were performed using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA).

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