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Detection of Intratumoral Aromatase in Breast Carcinomas

An Immunohistochemical Study with Clinicopathologic Correlation

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The expression of aromatase was evaluated in 38 breast carcinomas by an immunohistochemical method (ABC) using an specific polyclonal antibody against human placental aromatase. Fifteen tumors (40%) showed significant immunoreactivity, as defined by cytoplasmic positivity of moderate intensity present in at least 15% of the cells. The results were correlated with the estrogen and progesterone hormone receptor status and several clinicopathologic parameters such as age, tumor size, lymph node status, and stage of the disease. There was a significant, but inverse, correlation between the aromatase activity and the estrogen receptor status ($P = 0.04$), indicating the likelihood of negative estrogen if substantial aromatase activity was present. No statistically significant correlation was found between the presence of intratumoral aromatase and the rest of the parameters studied ($P > 0.7$). Nor was there a correlation between the aromatase content of the tumors and the menopausal status. The degree of intratumoral heterogeneity of the aromatase content was minimal in six cases where multiple samples from each tumor were analyzed. This is the first study reporting the detection of aromatase in archival material from breast carcinomas using immunohistochemical techniques. The lack of biologic significance of its presence in breast cancer reported here and by others using biochemical assays should be validated in larger series with longer follow-up. The method described can be readily used for that objective. (Am J Pathol 1992, 140:337–343)

Aromatase cytochrome P-450 plays a key role in estrogen biosynthesis. This enzyme catalyzes three hydroxylation reactions converting testosterone and androstenedione to estradiol and estrone, respectively, in various tissues. Examples of those include placenta, in which is essential to initiate the implantation of the ovum and the maintenance of pregnancy[1]; adipose tissue, an important source of estrogens in men and postmenopausal women[2,3]; hypothalamus, in which aromatase expresses transiently and is critical in the imprinting of sexually dimorphic metabolic and behavioral patterns[4]; testis[5]; normal breast[6]; and some breast tumors in which estrogens can be produced through intratumoral aromatase.[6,7]

Estrogens are implicated in the development of breast carcinomas.[8] It has been hypothesized that aromatase would contribute to their synthesis in the tumor and in the peritumoral adipose tissue, especially in postmenopausal women in whom the ovaries are no longer the main source of estrogens.[3,9] Some authors have found a significant correlation between intratumoral aromatization and tumoral response to the aromatase inhibitor aminoglutethimide.[10] The correlation between aromatase activity and estrogen (ER) or progesterone receptors (PR) using biochemical assays has been found to be negative by most investigators,[11] although some reports claim a positive relationship.[12]

Detection of aromatase by immunohistochemistry has been reported in the placenta,[13] ovary,[14] adrenal and adrenal tumors,[15] and brain,[16] but never in breast. Recently we have generated polyclonal antibodies against human placental aromatase.[17] We have investigated the existence of aromatase in 38 patients with breast carcinoma through semiquantitative analysis by immunohistochemical methods using anti-aromatase antibodies in an attempt to elucidate the biologic significance of tumor

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aromatization. Its presence was correlated with its measured activity and with the estrogen and progesterone receptor status and various clinical and histopathologic parameters.

Materials and Methods

Cases

Thirty-eight primary breast carcinoma samples were selected for the study from the files of the Division of Pathology at The City of Hope. The only criteria followed for the selection was that ER and PR measurement had been performed by a biochemical method, and that frozen tissue representative of the tumor was available for RNA extraction. The surgical pathology report and the medical records of the patients were reviewed to obtain the clinicopathologic information needed. Because all the cases were relatively recent, no significant follow-up was available.

Immunohistochemical Studies

The avidin-biotin complex method was used. Briefly, 3- to 5-μ-thick sections were cut from paraffin-embedded tissues and mounted in poly-L-lysine–coated slides. After deparaffinization and rehydration in xylene and ethanol, respectively, endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol. The tissues were digested with trypsin (0.1% in phosphate-buffered saline [PBS]) and incubated for 30 minutes with 5% normal goat serum in PBS. Rabbit aromatase antibodies (20 μg/ml), which preparation has been previously described,[17] were reacted overnight at room temperature with the tissues. Biotinylated anti-rabbit gamma G immunoglobulin (IgG) (1:100 in PBS for 30 minutes) was used as secondary antibody, which was followed by incubation with the peroxidase-linked avidin complex (1:80 for 1 hour) (Vectastains, Vector Laboratories, Burlingame, CA). The color was developed with 3,3-diaminobenzidine tetrahydrochloride (0.05% DAB in PBS for 10 minutes). Three washes in PBS buffer, pH 7.4, were done after each one of the incubations. The slides were counterstained with hematoxylin, rehydrated with increasing concentrations of ethanol, and mounted with Eukit (Am. Histol. Reapeut Co., Stockton, CA). Negative controls were done substituting P-450 aromatase antibody for an unrelated antibody and PBS. The results were evaluated by estimating the percentage of tumor cells with positive cytoplasmic stain, whose intensity was visually semiquantified from 0 (negative) to 3+ (maximum stain).

Estrogen and Progesterone Receptor Assays

On receiving the samples, generally within 15 minutes from tissue removal, the tumors were aliquoted. The portions assigned to the ER and PR biochemical assay were immediately snap frozen in liquid nitrogen and kept frozen at −70°C until they were sent for analysis. The estrogen receptor binding assay was performed by the Nichols Institute Labs., San Juan Capistrano, California, after the standard method in which aliquots of the cytosol are obtained by tumor homogenization and ultracentrifugation; the ER and PR content is determined by estradiol binding after removal of the unbound steroid with dextran-coated charcoal. Values of <3, 3 to 9, and >10 fmols/mg were interpreted as negative, equivocal, and positive, respectively.

Immunoblot Analysis of Human Placenta Microsomes

Human term placental microsomes were electrophoresed by dosium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli,[18] using a 5% to 15% gradient gel. The samples were electrophoretically transferred to nitrocellulose and probed with the anti-aromatase antibodies, followed by ^{125}I-labeled donkey anti-rabbit IgG (Amersham, Arlington Heights, IL). The blot was then exposed to x-ray film for 8 hours at −70°C.

Aromatase Expression Analyzed by Polymerase Chain Reaction

Total RNA was isolated from the tissue specimens following the method of Chirgwin et al.[19] Polymerase chain reactions (PCR) were performed according to the procedure of Saiki et al,[20] using the isolated RNA (100 ng per reaction) as templates. The reactions were initiated with AMV reverse transcriptase (2 units) and were followed by Taq polymerase (5 units) in the presence of two primers (0.2 μmol of each of the two primers) with sequences derived from aromatase cDNA, 5′-ATCTCTGGAGAGGAAACACTCATTA-3′, and 5′- CTGACAGAGCTTTCATAAAGAAGGG-3′ (reverse primer). The reactions were carried out for 40 cycles. The DNA products (198 bp) were analyzed in a 1.8% agarose gel, and the bands were visualized by ethidium bromide staining. The DNA products were transferred to Zetaprobe membranes (Biorad), followed by hybridization using a ^{32}P-labeled probe derived from the aromatase cDNA sequence in the region between the first two primers. The sequence of the latter oligonucleotide is 5′-ATTACAGCTCTCGATTCGGCAGCAA-3′. Hybridization using such a third probe further ensured that the PCR products are those expected. The conditions for prehybridization and hybridization were those recommended in the Biorad instruction manual.

Statistical Analysis

Correlation between the aromatase immunoreactivity and the menopausal status, age of the patient, hormonal receptor values, tumor size, presence of metastasis in lymph nodes, and stage of the disease were carried out using the Pearson chi-square test.

Results

The specificity of our antiserum against human aromatase was demonstrated by Western blot analysis. Figure 1 shows that the antibody reacts exclusively with a protein, which molecular weight corresponds to that of the aromatase in human placental microsomes. Additional bands that stain weakly are also present and they likely represent small amounts of enzyme aggregates that were formed during SDS PAGE. The amino acid sequence analysis of the aromatase preparation used was shown to be pure. Formalin-fixed and paraffin-embedded tissue sections of the human placenta were immunoreacted by immunohistochemistry, with the antibody demonstrating exclusive and intense staining on the syncytiotrophoblast of the chorionic villi (Figure 2a). When anti-aromatase antibody was replaced by irrelevant rabbit IgG, no immunoreaction was seen in the same tissue (Figure 2b). Additional tissues known to contain aromatase, such as the cortex of the adrenal gland, were also immunoreacted (Figure 2c). Similarly aromatase-negative tissues such as striated muscle and gastrointestinal mucosa also were evaluated, displaying no stain.

Of the 38 tumors studied, 15 showed positive intracytoplasmic immunoreactivity in at least 15% of the cells, with intensities that ranged between 2 and 3+. Two or more samples, corresponding to central and peripheral regions from the same tumor, were analyzed in six of the cases. No significant intratumoral heterogeneity for the presence of aromatase was seen in five cases. The aromatase activity varied, however, from very focal (<15%) with 2+ intensity, to focal (15% to 35%) with 3+ intensity in the two samples from the sixth tumor (Figure 2d). In one case, the epithelial cells from normal lobules stained positively while the adjacent tumor lacked detectable aromatase activity (Figure 3). Only on three cases peritumoral fat stained and never with the intensity seen on the tumors. One tumor displayed stromal staining that was less intense than that of the tumor cells. Tumors with weak reactivity in less than 15% of the cells were interpreted as negative. A microphotograph representative of the aromatase-negative breast tumors is displayed in Figure 4, showing minimal background activity typical of most samples.

As shown in Figure 5, aromatase RNA message was clearly detected in 3 of the 10 tumors that were analyzed using PCR. Two of the three were also positive by immunohistochemistry, and none of the PCR-negative tumors exhibited positive immunostain. These results provide additional evidence that the antibody reacts specifically

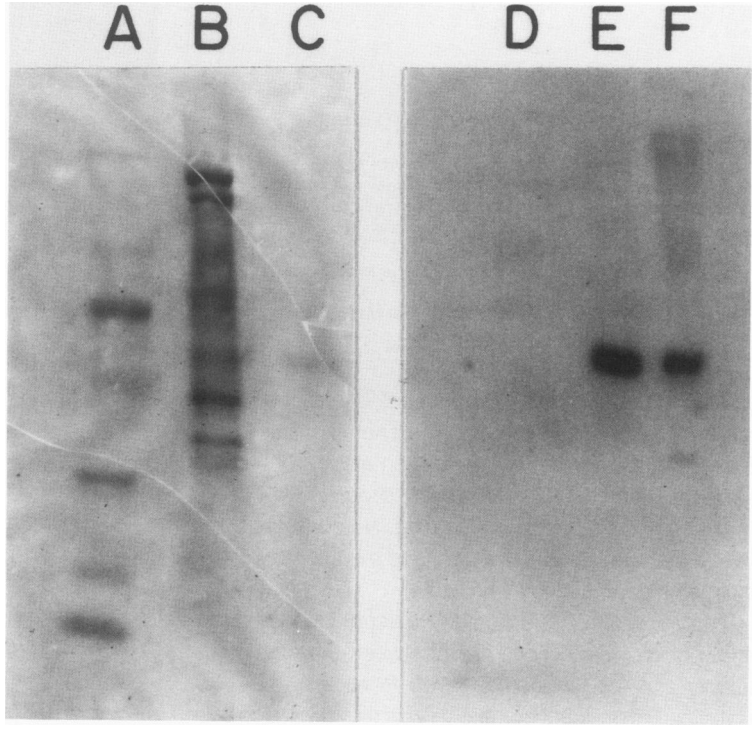

Figure 1. *Immunochemical identification of the aromatase protein in human placental microsomes.* **A,D:** *Protein molecular weight standards.* **B,E:** *Solubilized placental microsomes.* **C,F:** *Purified aromatase. (Left panel, coomassie blue stained gel; Right panel, Western blot analysis).*

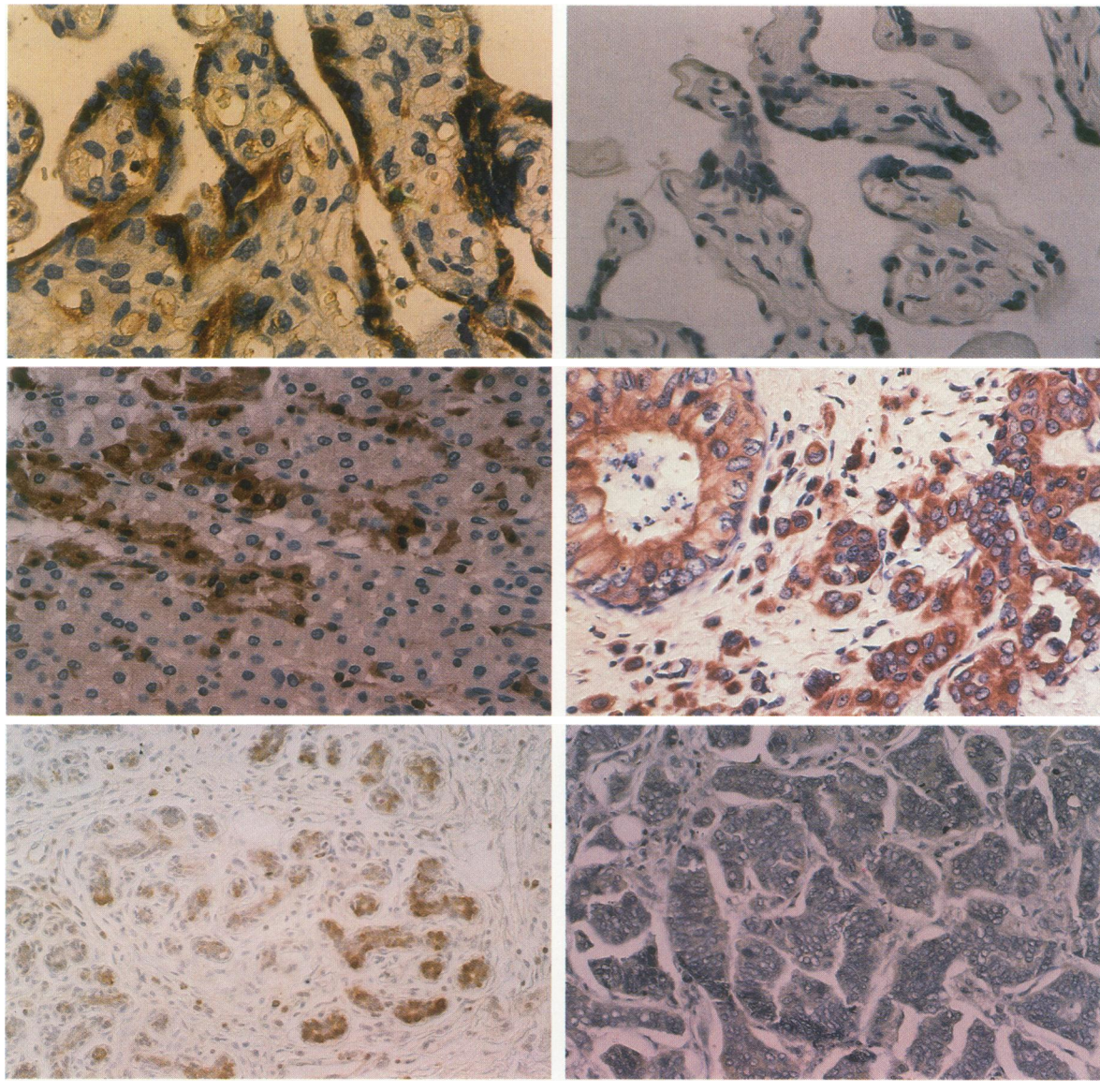

Figure 2. *Aromatase detection using immunohistochemical techniques (ABC) with the aromatase antiserum in formalin-fixed, paraffin-embedded tissue sections.* **A (top left):** *Human placenta showing intense staining on the syncytiotrophoblast (×400).* **B (top right):** *Same placenta in which antiaromatase antibody was replaced by irrelevant rabbit IgG.* **C (middle left):** *Section of a human adrenal displaying intense immunoreactivity on some of the cortical cells (×200).* **D (middle right):** *Moderately differentiated duct adenocarcinoma showing moderate to intense staining (×400).*
Figure 3 (bottom left). *Normal breast tissue with moderate aromatase expression that was adjacent to infiltrating ductal carcinoma lacing staining (×150).*
Figure 4 (bottom right). *Breast carcinoma not immunoreactive with antiaromatase antibody. The background stain present was minimal in most tumors studied (×150).*

with aromatase in the breast tumors, further confirming the specificity of the immunohistochemical detection. Sample 8 has significantly more aromatase mRNA than samples 5 and 10. A minor PCR product was seen, probably the result of a mispriming. No correlation between the strength of the signal and the intensity of the stain was seen.

Statistical analysis using the Pearson chi-square test showed an inverse correlation between the aromatase activity and the ER status, with a P value of 0.04. No statistically significant correlation was found with the size of the tumor, the presence of lymph node metastasis, or the stage of the disease ($P > 0.06$). Furthermore aromatase did not correlate with the menopausal status of the patients, as has been suggested (Table 1). Because of the short follow-up (maximum of 15 months), a correlation with the survival or the disease-free period was not possible.

Discussion

Aromatase has been implicated in the intratumoral production of estrogens by the aromatization of androgen

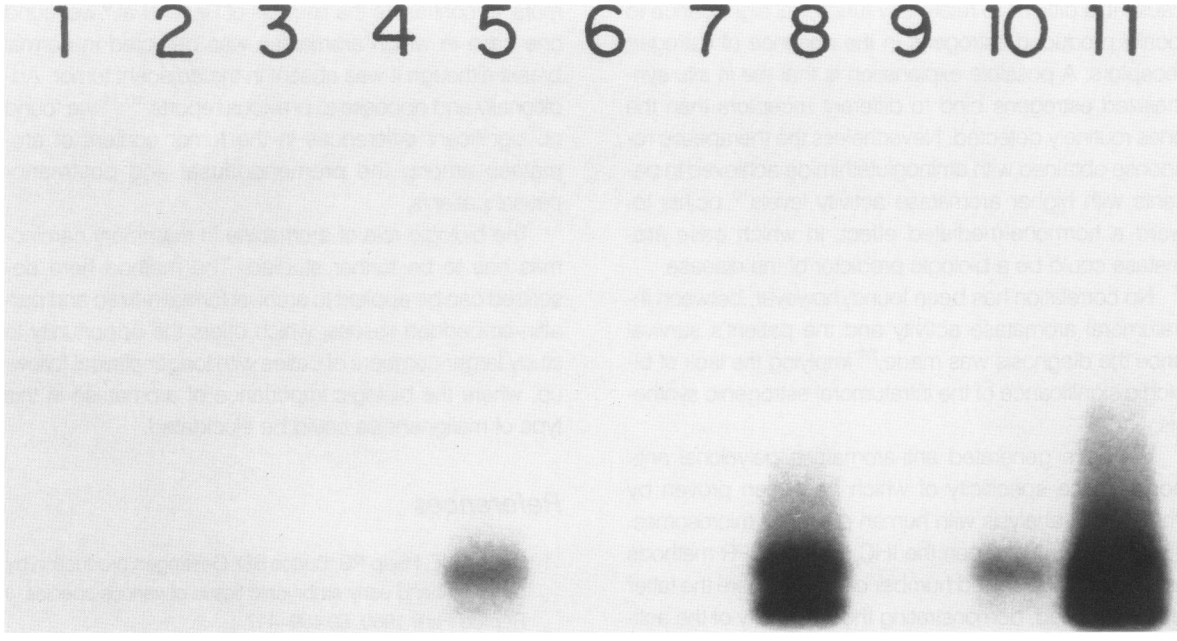

Figure 5. *Detection of aromatase mRNA by PCR method. The analysis was performed in 10 of the carcinomas included in the study (lanes 1–10) using placental RNA as a template. Notice the increased message present in cases 5, 8, and 10.*

precursors occurring in the majority of the mammary carcinomas[21–23] and adipose tissue adjacent to the tumors.[3] The contribution of the *in situ* estrogen synthesis to the total level of the hormone found in breast carcinomas is, however, controversial; although some authors have found it significant in most mammary carcinomas,[11] others have shown that its overall share was minimal[24–26] or only significant in some tumors.[6] Aromatization of breast carcinoma has been found to occur in greater degree in postmenopausal women,[10,11] where the most important source of estrogens, the ovary, is absent. The lack of correlation between intratumoral estrogen production, aromatase activity, and estrogen and progesterone receptors,[7,11,25] however, creates a mechanistic problem be-

Table 1. *Summary of the Breast Carcinoma Cases Studied*

	Aromatase negative	Aromatase positive	Total	*P* value*
Age				
Premenopause	9	5	14	0.72
Postmenopause	14	10	24	
ER				
Negative	3	6	9	
Positive	20	9	29	0.04
PR				
Negative	9	7	16	0.65
Positive	14	8	22	
Stage†				
I	7	2	9	
II	10	9	19	0.59
III	4	2	6	
IV	2	2	4	
Tumor size				
T_1	7	5	12	
T_2	10	6	16	
T_3	2	2	4	0.95
T_4	4	2	6	
Lymph nodes‡				
None	9	6	15	
1–3	5	3	8	0.97
>4	9	5	14	

* Analysis by the Pearson chi square.
† Following the classification (TNM) by the American Joint Committee on Cancer.
‡ Metastatic deposits to lymph nodes.

cause it is difficult to relate any functional significance to locally produced estrogens in the absence of estrogen receptors. A possible explanation is that the *in situ* synthesized estrogens bind to different receptors than the ones routinely detected. Nevertheless the therapeutic response obtained with aminoglutethimide achieved in patients with higher aromatase activity levels[10] points toward a hormone-mediated effect, in which case aromatase could be a biologic predictor of the disease.

No correlation has been found, however, between intratumoral aromatase activity and the patient's survival once the diagnosis was made,[24] implying the lack of biologic significance of the intratumoral estrogenic synthesis.

We have generated anti-aromatase polyclonal antibodies[17] the specificity of which has been proven by immunoblot analysis with human placental microsomes. The correlation between the IHC and the CPR methods was good in the limited number of cases where the latter was performed, demonstrating the specificity of the antibody and the reasonable sensitivity of the immunohistochemical method. IHC has the advantage of being a direct method detecting the enzyme itself instead of its activity. Furthermore the distribution of the enzyme within the tumor cells can be visualized and the assay can be performed in archival material. Its main disadvantage is that true quantification is not currently possible, although the microscopic evaluation used in this study provided consistent semiquantitative results. The PCR method is comparatively faster and very specific, assuming that the primers are appropriately chosen, but the need of fresh or properly fresh-frozen samples whose RNA has not been degraded limits the number of archival cases available for analysis. Both methods are direct and more sensitive than the measurement of the aromatase activity, and when used in combination should provide a very accurate determination of the aromatase expression. In this preliminary study, we have demonstrated that immunohistochemical techniques are reliable and sensitive to detect intratumoral aromatase. Our results evidence what others have shown by evaluating aromatase activity by biochemical methods: aromatase is present in a large number of breast carcinomas.[6,21,22] Furthermore, like Miller et al[12] and Silva et al,[24] we found in our small series no correlation between aromatase and some of the clinical parameters such as age, tumor size, stage, nodal status, etc. Because of the lack of significant follow-up, no statement can be done in relation to the survival; nonetheless its lack of correlation with the other parameters suggests that the survival will not be statistically significant, as proven by the same authors. A difference with previous studies is, however, the inverse correlation, albeit weak, that aromatase had, with the ER indicating a likelihood of detecting aromatase in the ER-negative tumors. In contrast to the findings of Reed et al,[6] we found one case in which aromatase was detected in normal breast although it was absent in the adjacent tumor. Additionally and opposite to previous reports,[10,11] we found no significant differences in the tumor content of aromatase among the premenopausal and postmenopausal patients.

The biologic role of aromatase in mammary carcinomas has to be further studied. The method here described can be applied to archival formalin-fixed and paraffin-embedded tissues, which offers the opportunity to study larger numbers of cases with longer clinical follow-up, where the biologic importance of aromatase in this type of malignancies could be elucidated.

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