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ER-NEGATIVE BREAST CARCINOMAS – AN IMMUNOPHENOTYPICAL STUDY

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ABSTRACT. Our aim was to study the morphological and immunohistochemical characteristics of a series of breast carcinomas, in comparative manner, between ER-negative and ER-positive tumours and to establish immunophenotypes of the ER-negative breast carcinomas. We established the morphological diagnosis and grade of 80-selected breast carcinomas on haematoxylin-eosin samples; additional sections were immunostained for AR, PSA, ER and PR, HER2/neu. The presence of lymphoid infiltrate, comedo-type necrosis, fibrosis and bizarre tumour giant cells were the most common morphological features in the ER-negative tumours. The apocrine character and the clear cells changes, squamoid cells changes, the medullary character and the adenoid cystic pattern were found only in the ER-negative carcinomas. We observed a significant correlation between the expression of ER and the grade of differentiation, PR, AR, PSA and HER2/neu expression; the majority of ER-negative carcinomas were poorly differentiated, PR-, and PSA-negative, but AR- and HER2/neu positive. The most frequent ER-negative immunophenotype in our study was the triple-negative phenotype (ER-/PR-/HER-) and the majority of these cases were AR-positive. All HER2/neu positive cases were also AR-positive. Our study supports the emerging studies that suggest a cross talk between steroids receptors, HER2/neu and PSA in breast carcinomas.

Keywords: estrogen receptor, androgen receptor, HER2/neu, prostate-specific antigen

INTRODUCTION

In Romania, about 6500 of women are diagnosed every year with breast cancer and 85% are diagnosed with advanced stages of disease; breast cancer incidence rates showed an increase from year 2000 with approximately 7% and about 2500 women die every year from breast cancer (Breast Cancer Facts&Figures, 2008).

Determination of ER status prior to therapeutic procedures has become a standard practice in the management of breast cancer and approximately 60-65% of carcinomas are ER positive. Estrogen receptor has proven to be a successful target for the treatment of breast cancer and consecutively ER-negative carcinomas benefit of fewer treatment strategies compared to ER-positive tumors (Pichon et al.1996).

ER negative breast carcinomas are a particular group of tumors usually poorly differentiated (grade 3), lymph node positive, that are generally thought to be aggressive with a decreased overall survival and with a poor prognosis. On the other hand, not all ER-negative breast carcinomas are poorly differentiated, for example adenoid cystic and secretory or apocrine carcinomas are ER- negative but well-differentiated (grade 1, 2) and their prognosis is generally good (Trendell-Smith et al. 1999, Rosen&Cranor, 1991). Moreover not all ER-negative and poorly differentiated carcinomas have a poor prognosis, for example pure medullary carcinomas that are poorly differentiated tumors have a better prognosis than the average (Jensen et al.1997).

Emerging data demonstrate that stratification of tumors by gene-expression profiles divides breast carcinomas into two main types and 5 subtypes, according to hormone receptor expression (negative and positive) and/or epithelial cellular origin (basal or luminal), with clinical implications. The hormone receptor-negative group has 3 subtypes: with HER2/neu overexpression, normal-like and basal subtype with positive epidermal growth factor receptor (EGFR), absent hormone-receptors and absent HER2 expression (triple-negative subtype) (Perou et al.2000, Sorlie et al.2001, 2003).

Our aim was to study the morphological spectrum and the immunohistochemical characteristics of a series of breast malignant tumors, in comparative manner between ER-negative and ER-positive tumors, to establish some immunophenotypes of the ER-negative breast carcinomas and to correlate them with the available clinicopathological data, in order to better understand the behavior of ER-negative breast carcinomas.

MATERIALS AND METHODS

We studied 80 selected specimens from female patients with breast cancer. All the specimens were

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surgically removed by mastectomy, at County Hospital in Timisoara, during 2004 year. The axillary lymph nodes were removed and the specimens were microscopically examined. The tumor size was expressed as the maximum diameter of the tumor as measured grossly and was confirmed microscopically. Clinical features of the patients were collected from the archives of the hospitals. Ethical approval was obtained and all patients gave informed consent.

The samples were formalin-fixed and paraffin-embedded, according to the routine procedure. From each representative paraffin block, we cut 4 μm sections. The pathological diagnosis and grading were established on hematoxylin-eosin samples and were based on the Standard recommendations by AFIP in 2004 and Elston and Ellis modified Scarff-Bloom-Richardson grading system. (Elston&Ellis, 1991). The hematoxylin-eosin sections were also examined for the various morphological parameters, such as appearance of tumor margins, the presence of lymphoid stromal infiltrate, secretory cell changes, apocrine character, comedo-type necrosis, squamoid or spindle cell changes, presence of tumor giant cells and prominent central fibrosis/necrosis, calcifications, adenoid cystic pattern, medullary character, vascular invasion.

Additional sections from each paraffin block were immunostained for AR, PSA, ER, PR, HER2/neu, using the avidin-biotin immunoperoxidase technique. Briefly, the slides were dewaxed and rehydrated and we blocked endogenous peroxidase using 3% hydrogen peroxide in deionized water. This step was followed by an antigen retrieval step, using microwave in sodium citrate buffer. The slides were incubated with the primary antibody. The dilutions and the specific features of the method for each primary antibody are summarized in the table 1. The secondary antibody (biotinylated antiserum) was applied; after washing with TBS (Tris buffered saline), we incubated the slides with freshly prepared avidin-biotin peroxidase complex for another 45 minutes. The final product of the reaction was visualized with 3, 3'-diaminobenzidine (DAB) and the nuclei were stained with Lillie's modified hematoxylin.

Table 1

Applied protocols and antibodies characteristics

Antibody against	Antigen retrieval	Primary antibody clone	Dilution	Incubation period with primary antibody	Working system
PSA	Microwave HIER* pH=6.6, 5-10 minutes	Dako polyclonal	Ready-to-use	30 minutes	EnVision
AR	Microwave HIER* at 95-99ºC, 25 min pH=9	Dako AR441	1:30	60 minutes	LSAB2
ER	Microwave HIER* at 90-99ºC, 20 min	Dako 1D5	Ready-to-use	30 minutes	LSAB2
PR	Microwave HIER* at 90-99ºC, 20 min	Dako PgR 636	Ready-to-use	30 minutes	LSAB2
HER2/neu	microwave HIER* (95-99º C), 40 minutes	Dako HercepTest polyclonal	Ready-to-use	30 minutes	EnVision

For semiquantitative evaluation of ER, PR and AR we considered the percentage of positive cells (samples were considered positive when at least 10% of nuclei were immunoreactive) and the intensity of the immunostaining according to the quick score method (Detre et al.1995). We quantified the results for PSA according to histoscore modified after Alanen (Alanen et al.1999), calculated from the estimated percentage of the PSA-positive cancer cells multiplied by the staining intensity category. For example, if 5% of the tumor cells showed moderate immunoreaction, the histoscore value was 0.05 x 2 = 0.1. The results were estimated as negative (-), weak positive (+1), moderate positive (+2) and strong positive (+3) in the following manner: 0 - 0.04 = negative; 0.05 – 0.74 = (+1); 0.75 – 1.4 = (+2); 1.5 – 3 = (+3). The staining intensity of human prostate epithelium used as a positive control was always stronger than that of the breast carcinoma cells. For the determination of HER2 overexpression we evaluated only the membrane staining as presence and intensity: the score (+2) was interpreted as weakly positive, (+3) as strongly positive and the scores 0 and (+1) were reported as negative (Ellis et al.2000).

Positive and negative controls were included in each staining batch. As positive control, we used 5 cases of prostate adenocarcinoma and prostate benign hyperplasia for AR and PSA and breast sections known to be positive for ER/PR. For HER2/neu we used Dako

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positive slides. Negative controls included sections processed in parallel, with omission of the primary antibody.

Statistical analysis. The frequency distribution of lymph node status, metastasis, histological type and grade, PSA, androgen receptor, progesterone receptor and HER2/neu status in ER-positive and ER-negative groups were compared using chi square test with odds ratio and 95% confidence intervals. A p-value of less than 0.05 was considered to be significant.

RESULTS AND DISCUSSIONS

Patients and tumor characteristics

All the patients were females with an age range of 23-80 years and a median of 53.5. Among the cases with known menopausal status, 18 were premenopausal and 35 postmenopausal, respectively. 62% of tumors were more than 2 cm in size. 41 (51.25%) of the cases were lymph node positive and 39 (48.75%) were lymph node negative. High proportions of the tumors were grade 2 and grade 3 (85%) and only 11 (16.6%) tumors were better differentiated (grade 1). The commonest histological types were ductal invasive (NOS), comprising 48/80 (60%) of the total cases. Other histopathological types were lobular invasive carcinomas 10/80, papillary (4) metaplastic carcinomas (5 cases), atypical medullary carcinomas (4), typical medullary carcinoma (2), adenoid cystic (2), DCIS (5) and 3 metastases (lymph node, skin and brain).

Table 2

Morphologic characteristics in ER-negative breast carcinomas

Morphologic characteristics	ER - (%)	ER + (%)
Pushing margin	3 (8.33%)	11 (23%)
Lymphoid infiltrate	19 (40%)	6 (16.6%)
Tumor giant cells	6 (12.5%)	0
Vascular invasion	6 (12.5%)	2 (5.5%)
Clear cells changes	6 (12.5%)	1 (2.7%)
Apocrine character	2 (4.16%)	0
Comedo-type necrosis	11 (23%)	1 (2.7%)
Fibrosis	13 (27%)	2 (5.5%)
Calcifications	4 (8.33%)	1 (2.7%)
Squamoid/spindle cells changes	5 (10.4%)	0
Adenoid cystic pattern	2 (4.16%)	0
Medullary character	5 (10.4%)	0

The presence of lymphoid infiltrate, comedo-type necrosis, and fibrosis were the most common morphological features seen in ER-negative tumors. The vascular invasion, clear cells changes and calcifications were features observed with a higher frequency in the ER-negative breast carcinomas. The apocrine character, squamoid and spindle cells changes, the medullary character, the adenoid cystic pattern and the bizarre giant cells were found only in the ER-negative carcinomas (*table 2*).

Immunohistochemical staining

The pattern of immunostaining for AR, ER and PR was nuclear. AR nuclear staining varied between individual tumor cells, but generally, it was of moderately and weakly intensity and heterogeneously distributed. PSA had a granular cytoplasmic pattern and HER2/neu had a membrane pattern. From the series of 80 carcinomas, 48 cases (57%) were ER-negative: 24/48 cases of ductal invasive carcinomas (50%), 2 cases of DCIS, 8/10 lobular invasive (80%), all 5 metaplastic carcinomas, the medullary carcinomas, the two adenoid cystic carcinomas and all 3 metastases. PRs were expressed in 19/32 (95%) of ER-positive and 5/48 (8.33%) of ER-negative carcinomas. ARs were expressed in 17/32 (53%) of ER-positive, respectively 45/48 (94%) of ER-negative carcinomas. HER2/neu was overexpressed in 2 (4%) of ER-positive and respectively in 11 (23%) from the ER-negative carcinomas. PSA was expressed in 25 (78%) of ER-positive and 9 (19%) of ER-negative breast cancers. All three metastases were ER-negative, AR-positive and overexpressed HER2/neu. The skin metastasis expressed also PSA.

Table 3

Statistical correlations of clinicopathological features in ER-negative and ER-positive breast carcinomas

Parameter	ER-negative	ER-positive	*p*
Menopausal status*	9	9	
Premenopausal (18)	25	10	0.12
Postmenopausal (35)			
Nodal status			
Positive (41)	28	13	
Negative (39)	20	19	0.3
Tumor size*			
≤2 (20)	15	5	
>2 (33)	19	14	0.19
Grade			
G1 (11)	5	6	
G2-3 (56)	44	12	**0.02****
PR			
Positive	5	19	
Negative	43	13	**0.00001****
HER			
Positive (13)	11	2	
Negative (67)	37	30	**0.04****
AR			
Positive (62)	45	17	
Negative (18)	3	15	**0.00001****
PSA			
Positive (34)	9	25	
Negative (46)	39	7	**0.00001****

** = only the known cases for this parameter; ** = statistical significant*

We observed a significant correlation between the expression of ER and the grade of differentiation (p=0.02), PR (p=0.00001), AR (p=0.00001), PSA (p=0.00001) and HER2/neu expression (p=0.04); the majority of ER-negative tumors were poorly differentiated, PR- and PSA-negative and AR and HER2/neu positive. We did not observe a statistically significant correlation with the tumor size, the nodal and menopausal status (*table 3*).

According to the expression of PR and HER2/neu, we established immunophenotypes of the ER-negative breast cancers and we correlated them with the immunohistochemical expression of AR and PSA and nodal status (*table 4*). The most frequent immunophenotype was ER-/PR-/HER- (triple negative tumors) with 34 cases out of 48 (70.8%); the majority of these cases were AR-positive (31 cases out of 48) but PSA-negative (30/48). All HER2/neu positive cases were also AR-positive, but only two from these cases expressed PSA. All the PR-positive cases were also PSA-positive. All cases ER-/PR-/HER+ (9 out 48 cases) were lymph node positive.

Table 4

Relationship between the ER-negative breast carcinomas immunophenotypes (ER/PR/HER2) and the expression of AR, PSA and nodal status

Immune Phenotype (48)	AR+ (45)	AR - (3)	PSA + (9)	PSA- (39)	N+ (28)	N- (20)
ER-/PR-/HER- 34 (70.8%)	***31***	3	4	**30**	15	**19**
ER-/PR-/HER+ 9 (18.75%)	**9**	0	0	**9**	**9**	0
ER-/PR+/HER-3 (7.7%)	**3**	0	**3**	0	2	1
ER-/PR+/HER+ 2 (5.13%)	**2**	0	**2**	0	2	0

Although the incidence of breast carcinomas is high, the overall mortality due to breast cancer is decreased, attributed to early application of various treatments. To reduce further the mortality from breast cancer, there is a desire to examine and characterize tumors of poor prognosis, to predict their biology, to ensure adequate therapy and to improve patients` outcome. There is also a need to develop additional forms of systemic, effective treatment in those tumors that fail to express known targets, such as estrogen and progesterone receptors or HER2/neu.

In the present study, the poorly differentiated carcinomas represented the majority of the cases but there were also exceptions. For example, adenoid cystic carcinomas are typically grade 1, ER-negative invasive breast cancer. The medullary carcinoma is a poorly differentiated carcinoma but it tends to have a lower frequency of axillary lymph node metastases than ductal types. Although 58% of ER-negative tumors were node-positive compared with only 41% in the group of ER-positive tumors, like other authors (Hayes et al.2001, Dowsett et al.2006), we did not obtain a statistically significant correlation for the lymph node status when we compared the two groups of tumors. For the tumor size either, we did not find a significant correlation. Scawn and Shousha (Scawn and Shousha 2002), reported lymph node metastasis in 46% of their cases and a wide range of tumor size, and they concluded that ER-status of the tumors is determined early in their natural history and supported the existence of two divergent pathways for the development of ER-negative and positive tumors. Regarding the lymphoid infiltration in ER-negative tumors, Iwao et al. (Iwao et al.2002) identified by gene-expression profiling that immunoglobulin genes are primarily expressed in ER-negative group. In the present study, we found lymphoid infiltration in 40% of ER-negative cases, compared with 16.6% of ER-positive tumors. The lymphoplasmacytic reaction was prominent especially in medullary carcinomas. Necrosis has been associated with higher incidence of node metastasis, higher mortality and has been related to the exceeding angiogenesis and rapid growth of the tumor (Jimenez et al. 2001). The fibrotic focus has been suggested to be a surrogate marker for hypoxia-driven ongoing angiogenesis (Hasebe et al. 2002). The presence of vascular invasion and increasing tumor grade has been shown to be independent prognostic factors (Pinder et al. 1994) and the absence of pushing margin was associated with the presence of vascular invasion (Putti et al. 2005). The present study shows necrosis in 23%, fibrosis in 27%, vascular invasion in 12.5% of ER-negative tumors, compared with 3%, 5.5% and 5.5% in ER-positive carcinomas, respectively. In ER-negative tumors we also found calcifications, clear cells, tumor giant cells, squamoid cell changes and apocrine character. The apocrine character was always associated with ER/PR negativity and AR and PSA positivity. We found a significant correlation for the ER expression with the tumor grade (p=0.02).

Immunohistochemical studies have shown that ARs are expressed in breast cancer with a higher frequency (70-90%) than ER (60-80%) and PR (50-70%) (Ellis et al. 1989, Kimura et al. 1993) and Agoff et al. (Agoff et al. 2003) reported that 49% of ER-negative tumors were AR-positive. Androgen receptors has been also correlated with longer survival (Isola 1993,Kuenen-Boumeester et al. 1996, Moinfar et al. 2003). Rakha et al. (Rakha et al. 2007) shown that androgen receptors may add important prognostic information in the lymph node-positive ER-negative tumors, and in addition to the traditional parameters (tumor size and nodal status) can be used to select low-risk patients in the time of

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primary surgery and can provide valuable information on treatment options especially in the subgroup with triple-negative phenotype. They emphasize the importance of routine staining of AR and basal cytokeratins in this class of carcinomas.

In our study, 45/48 (93%) of the ER-negative tumors expressed AR, compared with 17/32 (53%) in the group of ER-positive tumors. The difference was statistically significant (p=0.00001). We obtain also a significant correlation for the overexpression of HER2/neu: 23% of ER-negative and respectively 6.25% of ER-positive carcinomas expressed HER2neu (p=0.04). The most frequent ER-negative immunophenotype in our study was ER-/PR-/HER- (triple negative) and the majority of these cases was AR-positive, but did not express PSA. All the HER2/neu positive cases were also AR-positive, but only four from these cases expressed PSA.

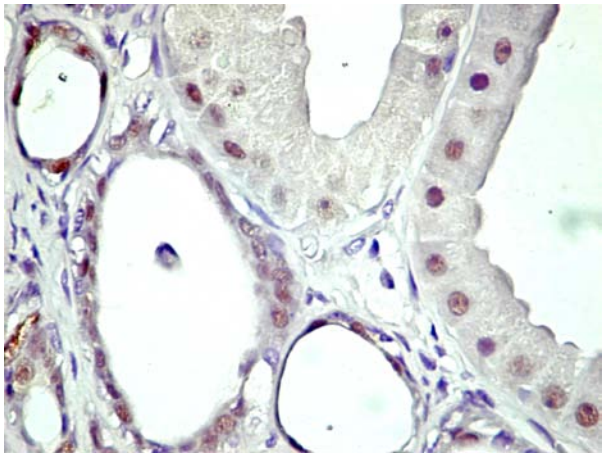

Fig. 1 Apocrine metaplasia with an intense expression of AR (AR IHC, x400)

It has been observed that the majorities of breast carcinoma skin metastasis were AR positive and ER/PR negative, so AR immunohistochemistry could serve as a marker for breast cancer in skin metastasis of unknown primary sites.

We investigated immunohistochemically only three breast carcinoma metastases, but in accordance with the findings of Bayer-Garner and colleagues (Bayer-Garner et al. 2000) these three cases displayed AR and loss of ER/PR expression. The skin metastasis was also PSA positive.

Elevated levels of PSA in breast tumors have been shown to be a favorable prognostic indicator in breast cancers, it were associated with AR and PR-positive tumors and it has been suggested that PSA may be a prognostic marker for patients with ER-/PR+ tumors. PSA values might identify a subset of estrogen-negative tumors, which could respond to endocrine therapy (Yu et al. 1996, 1998) but, up to now, the potential significance of PSA in breast cancer is still not defined. It has been suggested that PSA may act as a growth factor or a regulator of growth factors and it could be a marker of endogenous hormone balance between androgens, progesterone and estrogens (Black&Diamandis 2000, Foekens et al. 1999). These findings are in accordance with the findings of other authors which demonstrated that stimulation of AR and ErbB2 pathways leads to the cross-regulation of gene expression for *AR*, *ErbB2*, *FOXA1*, *XBP1*, *TFF3 and KLK3* (Naderi et al. 2007, 2008). Also, Magklara et al.2002 examined the expression of various known coactivators/corepressors proteins in different breast cancer cell lines. They found that the mRNA levels of steroid receptor coactivator 1, a known coactivator of the activation function 1 domain of the AR, were highest in the breast cancer lines with the greatest PSA production and lowest in the cell lines that secreted less PSA. This raises the possibility that the relative levels of specific coactivators/corepressors might differentially modulate AR transcriptional activity within the promoter/enhancer region of KLK3 (PSA) of different breast cancers' cell lines.

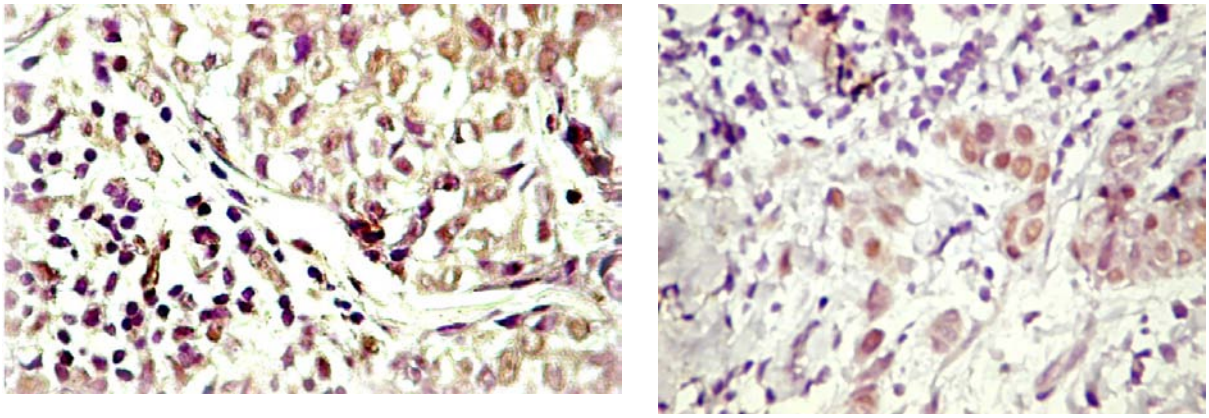

Fig. 2 a) Medullary carcinoma with a moderate positive immunoreaction for PSA in the tumor cells and negative for the lymphoid and conjunctive tissue (PSA EnVision, DAB, x400); b) Medullary carcinoma with an intense AR immunoreaction for the tumor cells and negative for the lymphocytes and components of the stroma (AR IHC, x400)

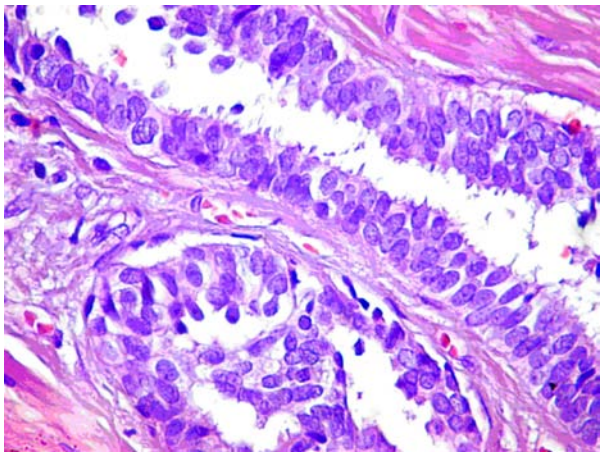

Fig. 3 DCIS with apocrine differentiation (HE, x400)

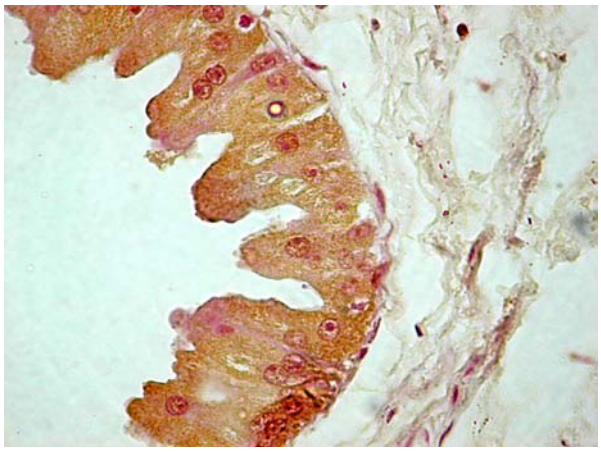

Fig. 4 Apocrine differentiation with an intense and homogenous reaction for PSA of epithelial cells, especially at the apical pole (PSA, EnVision, DAB, x400)

The data of the present study shows an expression of PSA in nine out 48 (18.75%) ER-negative cases compared with 25 out 32 (78%) ER-positive tumors; in another study, we demonstrated that the expression of PSA in breast carcinomas was correlated with AR and PR expression (Narita et al. 2008). In the present group of tumors that are predominantly ER-negative, all the ER-negative tumors that expressed PR were also PSA-positive; this fact indicates again the correlation between PSA and progesterone receptors.

On the other hand, in our cases, the lesions that displayed apocrine character expressed always AR and PSA and did not express ER/PR. This is in accordance with the findings of Mannello et al. 1996 that showed a high concentration of PSA in apocrine cysts and suggested that PSA may have a role in the malignant transformation. After Hall et al. 1998 positive immunoreactions for both PSA and GCDFP (gross cystic disease fluid protein)-15 were highly dependent on AR-status (98% of PSA-positive and 92% of GCDFP-positive tumors were AR-positive), but unrelated to age, ER/PR status and axillary's lymph node involvement. Apocrine epithelium is seen in a wide spectrum of breast entities, ranging from benign lesions to invasive carcinoma. Apocrine epithelium may reflect instability of the breast epithelium, creating an environment that could favor further oncogenic alterations and there are studies that support the idea that some benign epithelial apocrine lesions are clonal lesions and may be considered as pre-malignant (Zagorianakou et al. 2006). Farmer et al. 2005 have shown that androgen receptors expression level divides ER-negative breast tumors in two major gene expression clusters: ER-/AR- (basal) and ER-/AR+ (molecular apocrine) subtypes; they also shown that there is a higher HER2/neu expression in the apocrine subtype. Other studies have confirmed these findings, and demonstrate that the apocrine subtype has a gene signature similar to that of estrogen response; furthermore, a cell line model for the apocrine subtype demonstrates an increased proliferation in response to the androgen treatment, which can be reversed using the antiandrogen agent flutamide (Doane et al. 2006). The higher expression of ErbB2 in the apocrine subtype suggests a cross talk between AR and ErbB2 pathways in this subtype of breast cancer. Moreover, in prostate cancer, the ErbB2/ErbB3 pathways regulate AR by stabilizing AR protein levels and optimizing the binding of AR to the promoter/enhancer regions of androgen-regulated genes (Mellinghoff et al. 2004). We had a too little number of cases with apocrine differentiation, but in the present study, the expression of HER2/neu for the apocrine changes was as high as in the non-apocrine tumors; on the other hand, all the cases that overexpressed HER2/neu expressed also AR. This finding was in accordance with the results of Matsuo et al. 2002; this group has also reported that the rate of positive p53, HER2 and BCL-2 in apocrine carcinoma was almost the same as that of non-apocrine carcinomas.

CONCLUSIONS

The presence of lymphoid infiltrate, comedo-type necrosis, fibrosis and bizarre tumor giant cells were the most common morphological features observed in the ER-negative tumors. The apocrine character and the clear cells changes, squamoid cells changes, the medullary character and the adenoid cystic pattern were found only in the ER-negative carcinomas. We observed a significant correlation between the expression of ER and the grade of differentiation, PR, AR, PSA and HER2/neu expression; the majority of ER-negative carcinomas were poorly differentiated, PR-, and PSA-negative, but AR- and HER2/neu positive. We did not observe a significant correlation with the tumor size, the nodal and menopausal status. The most frequent ER-negative immunophenotype in our study was the triple negative phenotype (ER-/PR-/HER-), and the majority of these cases were AR-positive. All HER2/neu positive cases were also AR-positive. On the other hand, our study supports the emerging studies that suggest a cross talk between

steroids receptors, HER2/neu and PSA in breast carcinomas.

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