Expression of Androgen Receptors in Primary Breast Canser

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Abstract: Background: The objective of the study was to evaluate the prognostic effect of androgen receptor (AR) in breast cancers. **Patients and methods**: We investigated immunohistochemical AR expression from paraffin blocks of one hundred patients between 2007 and 2011, and analyzed demographics and outcomes using univariet analyses. Tumors with ≥10% nuclear-stained cells were considered positive for AR. **Results:** AR was expressed in 62% of patients. AR was significantly related to older age at diagnosis, smaller tumor size, histological type, higher positivity of hormone receptors and the administration of systemic treatment. In estrogen receptor (ER)-negative tumors, AR was distinctively associated with histological type and progesterone receptors unexpression. With a mean follow-up of 35.72 months, AR expression was a significant prognostic factor for DFS and OS in all patients. The 3-year DFS and OS of patients with AR-positive tumor were 87.1% and 90.73%, respectively. The 3-year DFS and OS of those with AR-negative tumor were 66.32% and 84.21%, respectively. AR expression was positively associated with survival outcomes in all patients. **Conclusions**: AR is significantly associated with favorable features in breast cancers and related to better outcomes in ER-positive not in ER-negative tumors. These results suggest that AR could be an additional marker for endocrine responsiveness in ER-positive cancers and a candidate for therapeutic targeting of ER-negative tumors.
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1. Introduction

Breast cancer is the most common malignancy in women in the United States and is second only to lung cancer as a cause of cancer death. The American Cancer Society estimates that 234,580 new cases of invasive breast cancer will be diagnosed and 40,030 will die of breast cancer in the United States in 2013[(1)].

In a population based cancer registries in Gharbia, Egypt, breast cancer was the most frequent cancer among Egyptian females. Breast cancer represented 17.5% of all incident cancers, accounting for 35.7% of all newly diagnosed female cancers. The crude incidence rate for females was 33.1/100,000 female population [(2).]

Traditional histopathological factors including tumor size, axillary lymph node metastasis and histologic grade, as well as new biomarkers including steroid hormone receptors and HER-2 are valuable as predictive and prognostic factors in breast cancer [(3,4)].

However, breast cancers have heterogeneous features. It is difficult to predict outcomes in all breast cancer patients using traditional histopathological factors and the same biomarkers. The validation of new emerging biomarkers is required to determine whether they are significantly beneficial for making a prognosis and guiding management algorithms [(5)].

The androgen receptor (AR) is one such newly emerging biomarker [(6)]. Many breast cancers express AR. Since AR belongs to the nuclear steroid hormone receptor family, it shows high structural, functional and topographic similarity to ER and PgR[(3,7,8)]. However, AR has not been well characterized in terms of its role as a predictive or a prognostic factor and the clinical significance of its expression in breast cancer patients remains unknown.

The aims of this study were to investigate the association between AR expression and clinicopathological parameters and to evaluate the implications of AR expression in patients with breast cancer, including stratified analyses by ER status.

2. Patients and Methods

We prospectively collected tumor tissues from specimens of surgically resected breast carcinoma at Clinical Oncology Department, Tanta University Hospital from January 2007 to December 2011. All tumor tissues were fixed in 10% buffered formalin and embedded in paraffin blocks.

One hundred patients with invasive breast carcinoma were enrolled. Patients with pure in situ carcinoma of the breast, recurrent or metastatic disease, bilateral breast cancers, or non-epithelial origin breast cancer such as phylloides tumor, sarcoma, or lymphoma, as well as those receiving neoadjuvant chemotherapy, were excluded.

Data regarding patient demographics, histopathology of the primary tumor, treatment patterns, and survival were retrospectively obtained by reviewing medical records. The type of surgery and

adjuvant therapies were determined not by AR expression but by international guidelines. Patients were treated with either mastectomy or breast-conserving surgery and sentinel lymph node biopsy or axillary lymph node dissection. After surgery, local radiotherapy or adjuvant systemic treatments were administered if the patient was able to tolerate it.

Hormonal therapy included either Tamxifen 20 mg /day, or Aromatase inhibitors 2mg/day according to menopausal status for ER and/ or PR +ve patients for 5 years. Clinical follow-up included history taking, physical examination, laboratory tests, and radiological imaging tests every 6–12 months for detection of relapse.

Tumor stage was based on the American Joint Committee on Cancer 6th edition criteria.

Procedures for immunostaining:

Immunohistochemical studies were performed using streptavidinbiotin method as described by Eissa and Shoman[9].

The examination was done on 10 % formalin fixed, paraffin embedded tissue blocks for evaluation of AR expression. The staging procedure was done as follow:

***Deparaffinization and rehydration of sections:**

Two sections from each block were cut on 4 micron thickness on adhesive positively charged slides. The slides were placed in xylene bath overnight to remove the paraffin. The sections were then rehydrated by placing them in descending grades of ethanol, followed by rinsing with distilled water after washing, the slides were dried around the tissue sections.

***Blocking endogenous peroxidase:**

Endogenous peroxidase activity was blocked by adding one to three drops of 3 % hydrogen peroxide in methanol solution to cover the sections. It was left for 30 minutes in room temperature. The slides were then rinsed by phosphate buffered saline (PBS) for 5 minutes and dried around the tissue sections.

***Antigen retrieval:**

Antigen retrieval was done by immersing the slides in citrate buffer solution (pH 6.0) and heating them in a microwave oven at 80 C for 4 minutes. This step was repeated three times. The slides were left to cool down to room temperature between each time, the sections were then rinsed by PBS and dried.

***Blocking non specific staining:**

Non specific blocking reagent (normal goat serum) was added to each section and left for 10 minutes at room temperature. Excess reagent was tapped off.

***Exposure to primary antibodies:**

The antibody for androgen receptor at dilution of this sections were then refrigerated at 4 ^{0}C overnight in humid closed chamber. The slides were then rinsed three times with PBS and dried.

***Exposure to biotinylated secondary antibody:**

The biotinylated secondary (link) antibody was applied to the sections. The slides were incubated in a humidity chamber at room temperature and left for 30 minutes. This was followed by rinsing in PBS three times and drying.

***Exposure to streptavidin – biotin complex:**

The enzyme labeled streptavidin – biotin complex was applied and incubated in a humidity chamber at room temperature for 30 minutes and the sections were rinsed with BPS three times then dried.

***Preparation of the working color reagent:**

The chromogen used was 3, 3 diaminobenzidine (DAB). One drop of DAB was added to each ml of buffered substrate. The components were mixed well and kept in a dark place.

***Colour development:**

The working color reagent was applied for 15 minutes then the sections were rinsed well with distilled water. The slides were then counterstained with hematoxylin and washed in running water Afterwards the sections were dehydrated in ascending grades of alcohol cleared twice in xylene then cover slipped with DPX.

The company that supplied the primary antibody and the dilution Factor was: AR Ab1 clone (AR441) is a mouse monoclonal antibody of immunoglobulin type (Kit no.9030. Labvision. UK) with dilution 1:50.

Pathologic review and scoring

All the stained slides reviewed for confirmation of diagnostic pathology cases were classified histopathologically according to the WHO classification of breast carcinoma [10] and graded according to [11] grading system which depend on the evaluation of the three tumor characteristics: tubule formation as an expression of glandular differentiation, nuclear pleomorphism and mitotic counts.

We considered nuclear labeling in ≥10% neoplastic cells as the cutoff point for positivity, similar to standardized criteria used for other steroid hormone receptors [12].

Local recurrence was defined as the reappearance of carcinoma in the treated remnant breast, skin, or chest wall. Events determining regional relapse were defined as recurrences to the ipsilateral axillary, supraclavicular, or internal mammary lymph nodes. Any recurrence at a distant site including the contralateral axillary or supraclavicular lymph nodes was considered to be distant metastasis.

Disease-free survival (DFS) time was measured from the date of the first curative surgery to

the date of the first locoregional or systemic relapse, or death without any type of relapse. Overall survival (OS) time was calculated from the date of the first definite operation to the date of the last follow-up, or death from any cause.

Statistical Analysis

Statistical analyses of categorical variables were performed using Pearson's chi-square test. The median duration of DFS&OS was calculated using the Kaplan- Meier method and group differences in survival time were investigated by a log-rank test.

P values of <0.05 were considered statistically significant. SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL) was used for all statistical analyses.

3. Results

Characteristics and outcomes in all patients

The age of patients ranged from 28 to 70 years. The mean age was (45.66± 15.31), ninety percent of patients were more than 35 years.

AR expression was demonstrated in 62% of patients. Positive expression of ER, PgR, and HER2 was observed in 74%, 80, and 22 of patients, respectively. The clinicopathological characteristics according to AR expression in all patients are summarized in Table 1.

Table 1: Patient characteristics & AR expression.

		AR						Chi-square	
		Positive		Negative		Total			
		N	%	N	%	N	%	X^2	*P*. value
Age	**<35**	2	3,22	8	21,05	10	10.00	6.456	0.011*
	>35	60	96,77	30	88,23	90	90.00		
T	**T1**	2	3.23	0	0.00	2	2.00	6.959	0.031*
	T2	50	80.65	24	63.16	74	74.00		
	T3	10	16.13	14	36.84	24	24.00		
N	**N0**	22	35.48	6	15.79	28	28.00	4.986	0.173
	N1	10	16.13	8	21.05	18	18.00		
	N2	24	38.71	18	47.37	42	42.00		
	N3	6	9.68	6	15.79	12	12.00		
M	**M0**	50	80.65	24	63.16	74	74.00	3.745	0.053
	M1	12	19.35	14	36.84	26	26.00		
Histology	**IDC**	54	87.10	32	84.21	86	86.00	5.822	0.05*
	ILC	4	6.45	6	15.79	10	10.00		
	Mixed	4	6.45	0	0.00	4	4.00		
ER	**Positive**	52	83.78	22	57.89	74	74.00	7.464	0.0063*
	Negative	10	16.12	16	42.10	26	26.00		
PR	**Positive**	55	88.70	25	65.76	80	80.00	24.903	<0.001*
	Negative	7	11,29	13	34.21	20	20.00		
HER2	**Positive**	14	22.58	6	15.79	20	20.00	0.679	0.410
	Negative	48	77.42	32	84.21	80	80.00		
TNBC	**Yes**	8	12.90	4	10.53	12	12.00	0.001	0.969
	No	54	87.10	34	89.47	88	88.00		
Surgery	**MRM**	50	80.65	28	73.68	78	78.00	0.665	0.415
	BCS	12	19.35	10	26.32	22	22.00		
Chemotherapy	**Done**	58	93.55	38	100.00	96	96.00	3.926	0.048*
	Not done	4	6.45	0	0.00	4	4.00		
Radiotherapy	**Done**	58	93.55	32	84.21	90	90.00	2.205	0.138
	Not done	4	6.45	6	15.79	10	10.00		
Endocrine therapy	**Done**	57	91.93	23	60.52	80	80.00	12.630	0.004*
	Not done	5	8.06	15	39.47	20	20.00		

Patients with AR-positive tumor showed a higher frequency of age over 35 years at diagnosis. A significant number of AR positive tumors were associated with older age, lower pathological tumor size and IDC type.

AR was significantly expressed in ER-positive and PgR-positive tumors. Among our study population, 70.3% (52 of 74) of patients with endocrine-responsive tumor (ERpositive and/or PgR-positive tumor) expressed AR.

No statistical difference was demonstrated in AR expression according to pathological nodal stage, distant metastases, HER2 overexpression, TNBC and locoregional treatment modalities. Patients with AR-negative tumor more frequently received adjuvant systemic chemotherapy, whereas those with AR-positive cancer were more often administered endocrine therapy.

With a mean follow-up duration of 35.72 months (SD 9.81 months), AR expression was a significant prognostic factor for DFS and OS in all patients (Figures 1 & 2). The 3-year DFS and OS of patients with AR-positive tumor were 87.1% and 90.73%, respectively. The 3-year DFS and OS of those with AR-negative tumor were 66.32% and 84.21%, respectively. AR expression was positively associated with survival outcomes in all patients.

Characteristics and outcomes stratified by ER status

The patient and tumor characteristics stratified by ER expression are shown in Table 2. AR expression was determined in 70.3 % (52 of 74) of patients with ER-positive breast cancer and in 38.5% (10 of 26) of those with ER-negative tumor.

In patients with ER-positive tumor, AR expression was significantly associated with old age, smaller tumor size and systemic treatment. In patients with ER-negative cancer, however, AR expression was statistically related to histological type and PR unexpression.

The DFS and OS according to AR expression in patients with ER-positive and ER-negative tumors, respectively, are presented in Figures 3-6.

In patients with ER-positive tumor, AR expression was positively associated with OS (Figure 5), as shown in all patients with statistical significance but no significant association with DFS.

However, in ER-negative breast cancers, there was no significant difference in either disease free or overall survival according to AR expression.

Table 2. Patient demographics stratified by estrogen receptor (ER) expression

		ER Positive					ER Negative				
		AR Positive		AR Negative		*P*-value	AR Positive		AR Negative		*P*-value
		N	%	N	%		N	%	N	%	
Age	**<35**	2	2.63	8	10.53	0.023*					
	>35	42	55.26	24	31.58		18	75.00	6	25.00	
T	**T1**	2	2.63	0	0.00						
	T2	38	50.00	22	28.95	0.020*	12	50.00	2	8.33	0.152
	T3	4	5.26	10	13.16		6	25.00	4	16.67	
N	**N0**	20	26.32	6	7.89		2	8.33	0	0.00	
	N1	6	7.89	8	10.53	0.075	4	16.67	0	0.00	0.121
	N2	14	18.42	12	15.79		10	41.67	6	25.00	
	N3	4	5.26	6	7.89		2	8.33	0	0.00	
M	**M0**	38	50.00	22	28.95	0.063	12	50.00	2	8.33	0.152
	M1	6	7.89	10	13.16		6	25.00	4	16.67	
Histology	**IDC**	40	52.63	28	36.84		14	58.33	4	16.67	
	ILC	4	5.26	4	5.26	0.634	0	0.00	2	8.33	0.019*
	Mixed						4	16.67	0	0.00	
PR	**Positive**	43	56.58	30	39.47	0.777	2	8.33	5	20.83	0.004*
	Negative	1	1.32	2	2.63		16	66.67	1	4.17	
HER2	**Positive**	4	5.26	4	5.26	0.634	10	41.67	2	8.33	0.342
	Negative	40	52.63	28	36.84		8	33.33	4	16.67	
Surgery	**MRM**	36	47.37	24	31.58	0.472	14	58.33	4	16.67	0.594
	BCS	8	10.53	8	10.53		4	16.67	2	8.33	
chemotherapy	**Done**	40	52.63	32	42.11	0.033*	18	75.00	6	25.00	
	Not done	4	5.26	0	0.00						
Radiotherapy	**Done**	40	52.63	26	34.21	0.222	18	75.00	6	25.00	
	Not done	4	5.26	6	7.89						
Endocrine therapy	**Done**	44	57,89	21	27.63	0.0001*	13	54.17	2	8.33	0.223
	Not done	0	0.00	11	14.47		5	20.83	4	16.67	

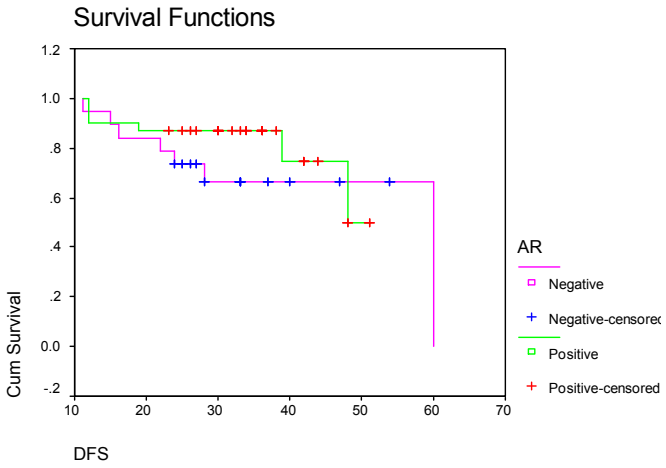

Fig 1:Disease free survival according to AR expression. Log Rank=3.86; P-value=0.04

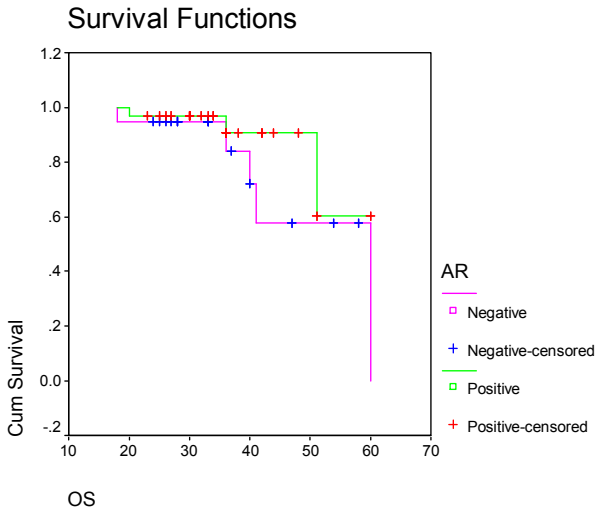

Fig 2: Overall survival according to AR expressionLog Rank=3.96; P-value=0.04

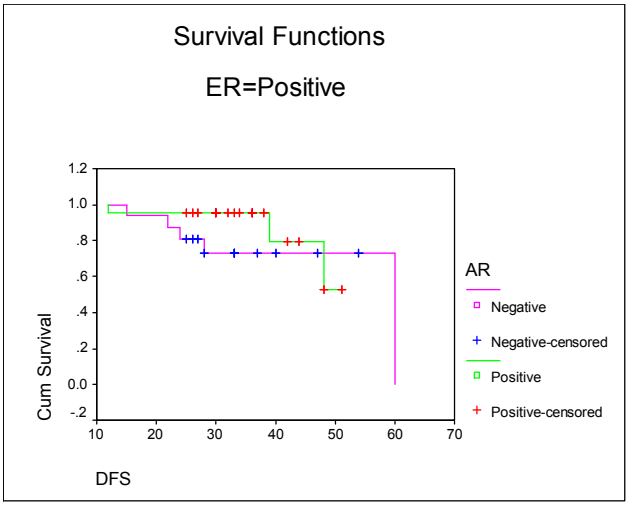

Fig 3: Disease free survival according to AR expression in ER positive patients. Log Rank=1.69 P-value=0.19

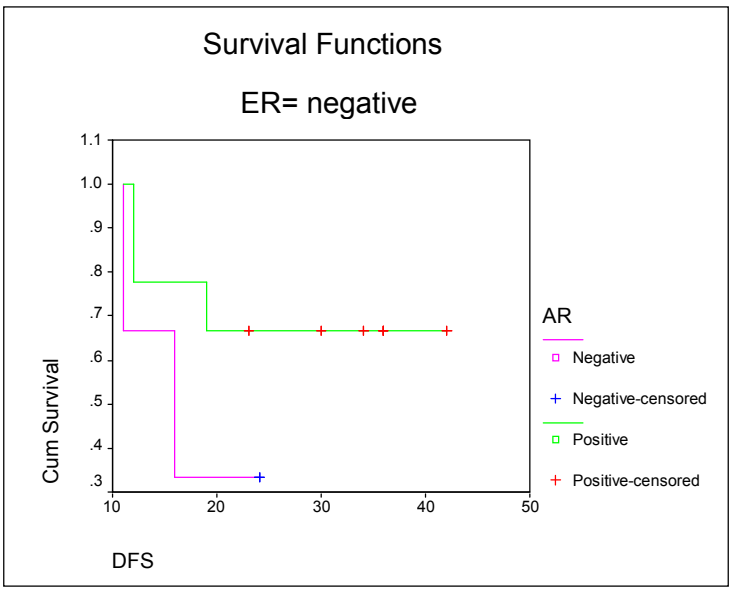

Fig 4: Disease free survival according to AR expression in ER negative patients. Log Rank= 3.09; P-value=0.07

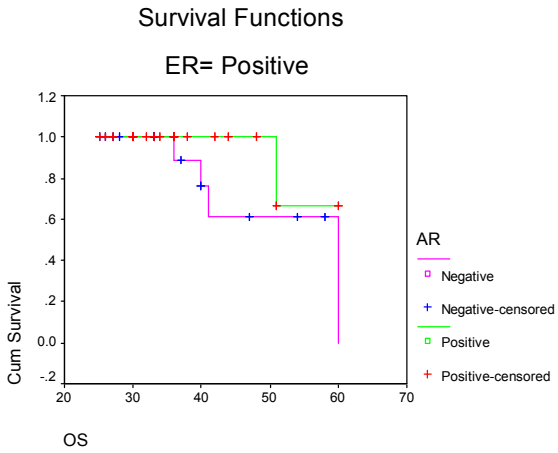

Fig 5: Overall survival according to AR expression in ER positive patients. Log Rank=4.50; P-value=0.03

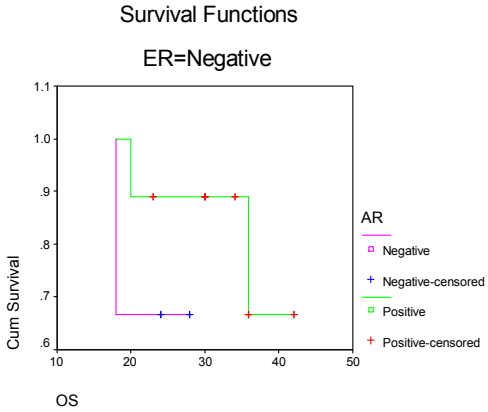

Fig6: Overall survival according to AR expression in ER negative patients. Log Rank= 2.01; P-value=0.15

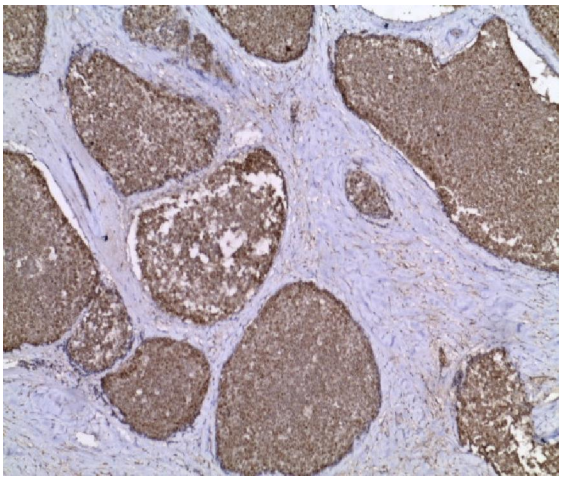

Fig. 7: Invasive duct carcinoma showing strong nuclear expression of AR in the malignant cells

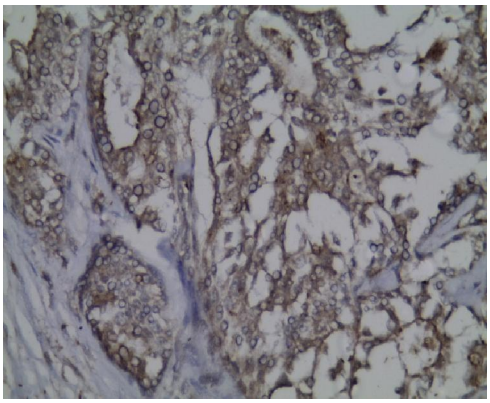

Fig. 8: Invasive duct carcinoma showing moderate nuclear expression of AR in the malignant cells

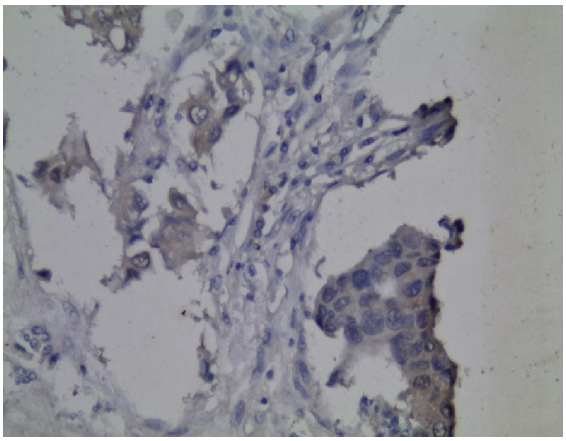

Fig. 9: Invasive duct carcinoma showing weak nuclear expression of AR in the malignant cells

4. Discussion

Breast cancer is a highly hormone-dependent tumor. The role of estrogen and its receptor is well established in the carcinogenesis and tumor progression; therefore, indirect or direct inhibition of ER pathways is the mainstay for treatment of breast carcinoma [13]. Although androgen is a dominant steroid hormone throughout a woman's life and a necessary precursor for estrogen biosynthesis, AR has only recently been considered as an emerging biomarker in breast cancer [14,15]. However, the clinical significance and functional role of androgen levels and of androgen receptor (AR) expression in breast cancer has not been well defined.

AR is a member of the larger nuclear receptor family that mediates the biological functions

of androgens [3]. Some biochemical and immunohistochemical data have indicated the presence of AR in breast cancer tissue. In particular, recent studies show that AR is expressed in a considerable proportion of breast cancers, ranging from 35 to 70% of cases and is frequently coexpressed with ER and PgR but rarely with HER2 [12,16].

The results of our study support these findings; in fact, we found AR expression in a high percentage (62%) of primary breast cancer patients & its expression was observed in significant levels in endocrine responsive tumors including ER+ve & PR+ve tumors (33% and 88% respectively). However, AR is also expressed in small number of ER-ve & PR-ve tumors (16% and 11% respectively). There was no significant interaction between AR and HER2 in all patients of this study. These data suggest that ARs are involved in the pathogenesis of at least a subset of breast carcinomas.

Yu *et al.,*[17] did not find a significant correlation between AR and age or menopausal status in their patients, in contrast with Luo*et al.,*[18] who found that AR expression correlated with increasing age. In our study, we could not find any tumor correlation of AR expression and the age of the patients.

It has been shown that AR is frequently expressed in some types of breast carcinoma, including apocrine or lobular carcinoma, and less expressed in other types such as mucinous carcinoma, [18] while Park *et al.,*[19] reported that among 301 AR+ve patients, higher positive rates of AR(88%) were shown in ductal type. Our study showed similar results. AR positivity of the ductal type was 87%. These results are somewhat limited by our small sample size.

Regarding the nodal status, which represents a reliable prognostic factor in breast carcinoma, there was no significant correlation between lymph node status and AR expression in our study. This is in agreement with other investigations [17] but in contrast with result of other studies [20] who found positive association between AR expression and low incidence of lymph node metastases.

Park *et al.,*[21] reported that a significant number of AR+ve tumors (535 from541) were associated with smaller tumor size, in agreement with our results, we found that 52 patients from 62 AR+ve patients were associated with smaller tumor size. However, other studies did not find any correlation between AR expression and pathological tumor size [17].

According to occurrence of distant metastasis, we did not find any statistically significant difference between AR expression and occurrence of distant metastasis, in agreement with Park *et al.,*[19].

Although we did not consider types or regimens of systemic therapies in this study, AR might provide an additional predictive role for systemic treatments including endocrine therapy because 85,5% of our ER positive subgroup received endocrine therapy &94,7% received chemo therapy. These results similar to other results [22,23].

Only a few studies have examined the association between AR expression and breast cancer survival, with some indicating improved survival among women with AR-positive tumors. [21]. In our study, we found that AR was a significant factor for survival outcome.

After controlling for well-established prognostic factors including ER status, however, AR expression was significantly associated with favorable features and related to better survival out comes in ER+ve patients, in agreement with results supported by Hu *et al.,*[24].

The few studies which have stratified by ER status do suggest that AR expression is associated with improved survival among women with ER-positive tumors [21].

Another retrospective study with a median follow-up period of 6.8 years also reported that AR expression is an independent prognostic factor of better outcome in patients with ER positive breast cancers [25].

Alternatively, Peters *et al.,*[26] recently reported that among 157 women with ER-positive invasive ductal breast cancer, patients with lower than the median percent (75%) of AR positivity in tumor cells, had a 3.0-folds increased risk of relapse and a 4.6-folds increased risk of cancer-related death in multivariate analysis. In functional analyses using breast cancer cell lines, Peters *et al.,*[26] demonstrated that AR and ER-α interact with one another and that AR can inhibit ER-α mediated growth of breast cancer cells. Thus, the AR is able to bind to estrogen responsive elements in ER-α and prevent activation of growth stimulatory effects.

The role of AR in ER-ve and triple negative breast cancer is not clear, with some studies reporting improved survival and others worse survival. Peters *et al.,*[26] found that among 58women with ER-negative breast cancer, no association between AR status and overall survival was observed. Similarly, Agoff*et al.,*[27] also found that AR expression in ER negative breast cancer (n=69) was not significantly associated with breast cancer survival in multivariate analyses, but this was attributed to the small sample size. On the contrary, Park *et al.,*[21] reported that AR is related to growth factor signaling in ER-negative tumors, and molecular apocrine tumors show a trend of poorer survival.

In our study, we did not find any association between AR expression with both the overall survival and the disease-free survival in ER-ve breast cancer patients.

Rakha *et al.,*[28] reported that in triple-negative tumors (n=282), especially those which were lymph node positive, absence of AR expression was associated with higher nuclear grade and increased development of recurrence and distant metastasis.

Luo *et al.,*[18] also found that the expression of AR was associated with higher 5-year disease-free survival in 137 triple negative breast cancer cases.

Another study of 97 women with triple negative breast tumors found that AR levels were not a significant prognostic factor for recurrence-free interval [29].

Currently, there are no available targeted therapies for women with triple negative disease. However, there are therapeutic targets of AR. Given that the triple negative subtype has the worst overall and disease free survival compared with other breast cancer subtypes [30], and more than one third of triple negative breast cancers are AR-positive, this represents a potential opportunity for novel targeted treatment for these women.

Bicalutamide is a non steroidal anti androgen therapy used to treat metastatic prostate cancer. A phase II trial of bicalutamide at a dose of 150 mg daily in patients with metastatic and ER−/PR−/AR+ breast cancers demonstrated that the 6-month clinical benefit ratio was 19%, indicating some activity in this select group of patients and supporting androgen blockade as a possible target in this disease. The median progression-free survival was 12 weeks. Bicalutamide was well tolerated with no grade 4/5 treatment-related adverse events observed [31].

Although there are no published studies of AR targeted therapy and breast cancer survival, taken together these data suggest that AR status may have a clinically important role in terms of prognosis and treatment for women with triple negative breast cancer [24].

In conclusion, our study confirms that ARs are expressed in most breast cancers. In addition, we demonstrated that AR positivity is associated with better outcome.

Since AR expression has important consequences on the prognosis and treatment of breast cancer, its presence should be precisely determined. Although we are still in the very early phase of clinical development, further studies of more cases and long-term prognostic valuation of different AR assays in patients comprising operable breast cancers should be carried out. The development of new strategies and drugs that can suppress or activate AR signaling will probably result in important clinical benefits.

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