Androgen Receptor Expression in Triple-negative Breast Cancer and its Relation with Epidermal Growth Factor Receptor, CD 105, and Clinicopathological Parameters

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Abstract

Background: Triple-negative breast cancers (TNBC) are the tumors lacking expression of estrogen receptors, progesterone receptors, and human epidermal growth factor 2. The highest level of androgen receptors (AR) expression belongs to the Luminal androgen receptor subtype. AR is expressed in 70 to 90% of primary breast cancers. The biological role of AR in breast cancer continues to emerge. The overexpression of epidermal growth factor receptor (EGFR) has been previously studied in TNBC, where it was found to be associated with poor prognosis. In the evaluation of neovascularization, CD105 (endoglin) was found to be superior to CD34 and CD31 owing to its greater affinity for endothelial cells in tumor-related angiogenic tissue. We conducted the present work to assess the expression profile of androgen receptor in TNBC cases and its correlation with other clinicopathological parameters, EGFR and CD 105, in order to evaluate its clinical significance.

Method: This retrospective study included 50 histologically confirmed breast cancer patients who were proven to be triple-negative based on immunohistochemical study. Formalin-fixed tissue blocks with tumor were chosen for immunohistochemical staining for AR, EGFR, CD105, and Ki 67.

Results: Positive AR expression was associated with older age, postmenopausal status, negative nodes, and grade II tumors. AR was inversely correlated with EGFR, while there was no correlation between AR and both Endoglin and Ki 67.

Conclusion: AR-positive TNBC may be a subtype of breast cancer with unique characteristics that could make it ideal for antiandrogen endocrine therapy. EGFR and Endoglin's distinct expression indicated that they might be unique biomarkers for targeted therapy and prognosis.

Keywords: Breast neoplasms, Androgen receptors, Immunohistochemistry

Introduction

Triple-negative breast cancers (TNBC) are defined as tumors lacking expression of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor 2 (HER-2). Depending on the thresholds used to define ER and PR positivity and the methods employed for HER2 assessment, these tumors account for 10%-17% of all breast carcinomas.[1] The main feature of TNBC, which have emerged from their similarity to basal-like cancers, is being significantly more aggressive than tumors of other molecular subtypes.[2] This aggressiveness is best illustrated by the fact that the risk of recurrence usually peaks between the first and third years and that most deaths occur in the first five years following the therapy.[3]

TNBC is a heterogeneous disease that encompasses distinct intrinsic molecular subtypes. Recently, studies of gene expression profiles have further stratified the TNBCs into six subtypes, expressing several different molecular markers unique to the different groups with differential potentials of aggressiveness.[4]

The luminal androgen receptor (LAR) subtype is enriched for hormonally regulated pathways and depends on the signaling of the androgen receptor (AR).[5] Even though AR may be expressed in multiple molecular TNBC subtypes, the LAR subtype has the highest level of AR expression.[6] The LAR subtype is a novel TNBC subtype with a distinct prognosis that provides an opportunity for the development of targeted therapy.[7]

AR is a member of the receptor family of nuclear steroid hormones that also includes ER and PR. Steroid hormone receptors are critical components of signaling pathways and play an important role in controlling gene expression as transcription factors. AR is expressed in 70 to 90% of primary breast cancers, a frequency comparable to or higher than either ER or PR. Meanwhile, ER and PR are widely recognized for their predictive and prognostic roles in breast cancer, the biological role of AR in breast cancer continues to emerge.[9] Increasing evidence supports the function of androgens and AR in breast cancer

pathogenesis, yet the role of the AR pathway in TNBC remains uncertain.[10]

The overexpression of epidermal growth factor receptors (EGFR) was has been previously studied in breast cancer. It occurs more commonly in TNBC, where it has been found to be associated with poor prognosis.[11,12] Several studies have investigated the prognostic importance of EGFR protein expression, gene copy numbers and mutations in breast cancer.[13,14] However, studying EGFR alterations at different molecular levels has not been reported for TNBC. Due to the high rate of overexpression of EGFR in TNBC, EGFR inhibitors are among the targeted agents being developed for TNBC treatment.

Concerning solid tumors, microvascular density (MVD) has become an essential component for determining angiogenesis. Pan-endothelial markers, including CD31, CD34, and von Willebrand (vWF) have been utilized for historical assessments of MVD for tumors.[15,16] These markers are specific to all endothelia and not just target the vascular endothelium of the tumor. Regarding the evaluation of neovascularization, CD105 (endoglin) was found to be superior to CD34 and CD31 since it has a greater affinity for endothelial cells in tumor-related angiogenic tissue, whereas CD34 and CD31 have a nonspecific reaction to normal and pathological vessels.[17,18]

The present work aimed to assess the expression profile of AR in TNBC cases and its correlation with other clinicopathological parameters, EGFR and CD 105 to evaluate its clinical significance.

Patients and Methods

This retrospective study included 50 histologically confirmed breast cancer patients presented to the Department of Cancer management and research, Medical Research Institute, Alexandria University, Egypt from January 2016 to March 2017.

All of the selected breast carcinoma patients were proven to be triple-negative (ER negative, PR negative, and Her2 negative) based on

immunohistochemical study performed in the Pathology Department, Medical Research Institute, Alexandria University, Egypt. HER2 staining of 2+ score by IHC with no gene amplification was verified with fluorescence in situ hybridization (FISH).

All the patients underwent surgical treatment either modified radical mastectomy or conservative breast surgery with axillary clearance. Clinicopathological parameters, including age, menopausal status, histologic grade and subtype, lymphovascular invasion, presence of axillary lymph node metastasis, and distant metastatic status were collected retrospectively. The patients with metastatic disease at presentation and those with non-invasive breast cancers were excluded from the study.

The study was approved by the Medical Research Institute's ethical commission (IORG#:IORG0008812) and according to the Helsinki declaration.

Immunohistochemical evaluation

For AR, EGFR, CD105, and Ki 67 immuno-histochemical staining, the archived formalin-fixed tumour tissue blocks were selected. Paraffin embedded tissue sections were cut at 3-5 micrometers thick, dried, deparaffinized, and rehydrated employing standard procedures. Endogenous peroxidase activity was blocked by 3% H2O2 incubation to avoid non-specific antibody binding. Antigen recovery was carried out by heating in a citrate buffer (pH 6.0) microwave oven in a thermo-resistant container for 15 minutes. We cooled the slides in buffer down to room temperature for 20 minutes, then, washed them twice in phosphate buffered saline. Immunocytochemical reaction was performed using the following antibodies:

  * Monoclonal mouse antibody against AR (Dako; Denmark),

  * Monoclonal mouse antibody against EGFR (BioSB; USA),

  * Polyclonal rabbit antibody against Endoglin (CD105) (Bio SB;USA),

  * Monoclonal mouse antibody against Ki 67 (Dako; Denmark).

The examined parts were incubated overnight

Table 1. Clinicopathological characteristics of the studied patients (n= 50)

Clinicopathologic variable	No.	%
Age (years)		
<45	14	28.0
≥45	36	72.0
Menopausal status		
Pre-menopausal	14	28.0
Post-menopausal	36	72.0
Family history		
Negative	36	72.0
Positive	14	28.0
Histologic subtype		
Invasive ductal NST	42	84.0
Invasive lobular	4	8.0
Invasive medullary	4	8.0
LN status		
Negative	28	56.0
Positive	22	44.0
Grade		
I	0	0.0
II	30	60.0
III	20	40.0
Tumor Size		
≤2cm	16	32.0
>2cm	34	68.0
Lymphovascular invasion		
Positive	32	64.0
Negative	18	36.0

Invasive ductal NST: Invasive ductal no special type; LN status: lymph nodes status

at room temperature with the antibodies. The incubation with secondary antibodies (30 minutes at room temperature) was subsequently reported. The slides were rinsed in phosphate buffered saline (PBS) (pH 7.0) three times, three minutes each, between each of the previous steps. We employed diaminobenzidine tetrahydrochloride (DAB) as a chromogen, applied to the slides for 5-15 minutes in the dark at room temperature in order to detect the obtained products.

All the sections were counter stained with hematoxylin, mounted, and examined using the light microscope. Prostate carcinoma was utilized as a positive control for AR, squamous cell carcinoma for EGFR, and renal cell carcinoma for CD105. The immunostaining results were assessed semi-quantitatively and reported as positive for AR, EGFR, and CD105 once more than 10% of the cells had nuclear immunostaining in a tumor. Ki 67 immunostaining was considered

positive, if there were nuclear staining in more than 20% of the tumor cells.[19]

Statistical analysis

The obtained data were analyzed with SPSS software package version 20.0. (Armonk, NY: IBM Corp). The qualitative data were described using numbers and percentages. The Kolmogorov-Smirnov test was utilized to verify the normality of distribution. The mean and standard deviation were calculated for quantitative variables. For the qualitative variables, we calculated the frequency and percentage. Chi-square test was performed in order to determine the association between clinicopathological parameters and expression of AR and between AR expression and other studied markers. Student t-test or Mann witney test were applied to compare the differences concerning the means among the groups.

The significance of the obtained results was judged at 5% level.

Results

Table 1 summarizes the clinicopathological characteristics of the studied patients. The mean age of the studied cases was 50.8 ± 10.17 years and the median age was 43.

Immunohistochemical characteristics of the studied cases

Table 2 represents a summary of the immuno-histochemical findings of the present work.

Relationship between AR expression and clinicopathological parameters of the patients

AR positive immunostaining showed an association with older age ($\geq$45years) ($P<0.001$), menopausal status ($P<0.001$), histopathologic subtypes ($P=0.008$), LN status ($P=0.001$), and tumor grade ($P=0.005$). Positive AR expression was associated with the age range of equal or over 45 years, postmenopausal status, negative nodal status, and grade II tumors.

Meanwhile, there was no statistical significant difference observed between AR immunostaining and family history, lymphovascular invasion and tumor size (Table 3).

Relationship between AR and the studied immuno-

Table 2. Distribution of the studied cases according to immuno-histochemical findings (n= 50)

Parameter	No.	%
AR		
Negative	28	56.0
Positive	22	44.0
EGFR		
Negative	30	60.0
Positive	20	40.0
Endoglin (CD 105)		
Negative	34	68.0
Positive	16	32.0
Ki 67		
<20%	34	68.0
$\geq$20%	16	32.0

AR: Androgen receptor; EGFR: Epidermal growth factor receptor

histochemical parameters

AR was inversely correlated with EGFR, where AR was significantly expressed in EGFR negative cases, while there was no correlations between AR and both Endoglin and Ki 67.

Discussion

The present study revealed that the frequency of AR immunohistochemical staining in TNBC patients was 44%, with a cut-off of 10%. There was a statistically significant association between AR status and the patient's age, menopausal status, and histopathologic subtype. AR staining was significantly associated with low grade and absence of lymph node metastasis. No statistically significant associations were observed between AR immunostaining and tumour size or lymphovascular invasion. Regarding the other biological markers, AR immunostaining was significantly correlated only with EGFR. Meanwhile, no statistical correlations were found between AR and Endoglin or Ki 67.

A significant variability exists in the reported literature regarding the frequency of AR expression in TNBC ranging from 6.6 to 75%.[20, 21] This heterogeneity results primarily from the variability in the number of patients included in the reported studies and the cut-off used for AR positivity (1% or > 10%). AR expression was 74.8% in ER-positive tumors and 31.8% in ER-negative tumors in one of the largest systematic

Table 3. Relationship between AR and clinicopathological parameters

Parameter	AR				χ^2	P
	Negative (n=28)		Positive (n= 22)			
	No.	%	No.	%		
Age (years)						
<45	14	50.0	0	0.0	15.278*	<0.001*
≥45	14	50.0	22	100.0		
Menopausal status						
Pre-menopausal	14	50.0	0	0.0	15.278*	<0.001*
Post-menopausal	14	50.0	22	100.0		
Family History						
Negative	18	64.3	18	81.8	1.878	0.171
Positive	10	35.7	4	18.2		
Histologic subtype						
Invasive ductal NST	24	85.7	18	81.8	7.578*	0.008*
Invasive lobular	4	17.3	0	0.0		
Invasive medullary	0	0.0	4	8.2		
LN status						
Negative	10	35.7	18	81.8	10.628*	0.001*
Positive	18	64.3	4	18.2		
Grade					7.792*	0.005*
I	0	0.0	0	0.0		
II	12	42.9	18	81.8		
III	16	57.1	4	18.2		
Tumor Size						
≤2cm	10	35.7	6	27.3	0.403	0.525
>2cm	18	64.3	16	72.7		
Lymphovascular invasion						
Positive	17	60.7	15	68.2	0.320	0.405
Negative	11	39.3	7	31.8		

AR: Androgen receptor; LN status: lymph node status; χ^2: Chi square test; P: P-values for comparing between the two groups
*: Statistically significant at $P \leq 0.05$

reviews which included 7693 breast cancers from 19 trials.[22]

In the current study, 72% of cases were in post-menopausal. This is in agreement with the findings of Akhtar et al. in their study on Indian women, in which 58.8% of the subjects with TNBC were post-menopausal.[23] We found that positive AR expression was significantly associated with postmenopausal status and invasive ductal carcinoma subtype. Teoh PY et al. estimated 85% of TNBC cases in their study had invasive ductal carcinoma, not special types although, in contrast with our findings they did not find any associations between menopausal status and AR positivity.[24]

In this study, 72% of TNBC patients were equal or over the age of 45 years compared with only 28% who were under 45 years of age. The mean age of the studied cases was 50.8 ± 10.17. This suggests that while TNBC is more common in younger age groups, a significant percentage of older patients still develop TNBC.

This has been agreed upon by Teoh PY et al., who estimated that the mean age of diagnosis of TNBC was 58.4 years.[24] In another study by Aapro M et al., TNBC represented in 18.4% of all breast cancers in patients aged ≥70 years.[25] This pattern has not been identified; however, in a research in western countries, TNBC has appeared in a younger age group.[3] Furthermore, we found that AR immune-reactivity was significantly related to the patient's age (P<0.001). This is in agreement with Samaka et al., who correlated AR with age in breast carcinoma patients.[26]

Regarding the tumor grade in our study, 60% of the cases were of grade 2 and 40% were of grade 3. Moreover, there was a statistically significant association between AR staining and lower grades. This is in agreement with

Table 4. Relationship between AR with EGFR, Endoglin and Ki67

Parameter	AR				χ^2	P
	Negative (n=28)		Positive (n= 22)			
	No.	%	No.	%		
EGFR						
Negative	8	28.6	22	100.0	26.19*	<0.001*
Positive	20	71.4	0	0.0		
Endoglin (CD 105)						
Negative	16	57.1	18	81.8	3.447	0.063
Positive	12	42.9	4	18.2		
Ki 67						
<20%	20	71.4	14	63.6	0.344	0.558
≥20%	8	28.6	8	36.4		

AR: Androgen receptor; EGFR: Epidermal growth factor receptor; χ^2: Chi square test; P: P values for comparing between the two groups
*: Statistically significant at $P \leq 0.05$

Rampurwala M et al., who showed that positive AR immunostaining is a favorable prognostic factor and associated with a lower clinical stage, lower histologic grade, and lower mitotic score.[27]

In the current study, 44% of the cases demonstrated positive lymph node metastasis compared with 56% with negative lymph node metastasis. AR immunostaining was inversely related to lymph node metastasis being positive in 81.8% of the patients with no lymph node metastasis. In a study by Rakha et al., there was also a significant association between AR expression and lymph node involvement (P=0.03).[28]

Unlike our results, other studies have indicated the lack of a significant relationship between AR expression and lymph node involvement.[29,30]

The tumor size in this study was ranged from 1.5 to 7 cm in the greatest dimension with 68% of cases having tumor sizes over 2 cm compared with only 32% of them with less than or equal to 2 cm-tumors in the greatest dimension.

This is in agreement with Qui J et al., who compared TNBC to non-TNBC and concluded that TNBC had a greater proportion of cases with tumors approximately 5 cm or more in the greatest dimension.[31]

In our study, AR was positive in 72.7% of the tumors over 2cm. Meanwhile, we found no statistically significant relations between tumor size and AR immunostaining. On the other hand, Zakaria et al. concluded that AR expression was significantly related to a smaller tumor size.[32]

Moreover, in this study AR immunostaining was not correlated with lymphovascular invasion. Gonzalez et al. also found no significant correlations between AR expression and lymphovascular invasion.[33]

The fact that TNBC is a heterogeneous disease that encompasses different intrinsic molecular subtypes with the overlap of AR and molecular apocrine signatures could explain this contradiction.

In TNBC, the EGFR is frequently overexpressed. Unfortunately, in patients with metastatic TNBC, several clinical trials have attempted to target EGFR, but failed to demonstrate a significant benefit.[34,35] In the current research, 40% of the cases were positive for EGFR, which is again in accordance with the literature.[36,37] In the adjuvant setting; however, there is no definitive evidence regarding the impact of EGFR expression on patients' outcome.[38]

We found a statistically significant inverse correlation between EGFR and AR immunohistochemical staining being higher in AR negative cases; this is in agreement with the results of Zhimin J et al.[39]

In solid tumors, CD105 has been found to be upregulated in the peri- and intra-tumoral blood vessel endothelial cells and in the stromal components of several types of cancer. Studies have depicted that increased MVD, as measured with a CD105, is associated with worse overall and disease-free survival.[40,41]

In the current study, 32% of the cases were positive for Endoglin. This is in agreement with the findings of Lopes et al., who concluded that the endoglin present in all the breast cancer subtypes and the number of immunopositive vessels was higher in the basal-like subgroups compared with the other molecular subsets.[42] Their findings suggested that the universal expression of MVD plays an important role as a target for anti-angiogenic therapy that is suitable for all tumor subsets. Nonetheless, as an anti-angiogenic treatment, targeting endoglin, could not be considered as a therapy specifically for triple-negative subset of breast carcinomas.

In our study, there was no statistically significant correlation between AR and Endoglin. Mishra et al. reported a statistically significant correlation between AR and Endoglin in locally advanced breast cancer.[43] The contradiction may be explained by the fact that the former study was not carried out only on TNBC, but included hormone positive cases as well.[43]

Ki67 is a nuclear protein widely used as a proliferative marker and its expression varies throughout the cell cycle with the highest expression during mitosis. In breast cancer, this protein has been extensively studied as a predictive and prognostic marker, although to date, there is no standard cut-off definition.[44]

In this investigation, 16 out of the 50 studied cases exhibited Ki67 positivity in 20% and more of tumor cells ' hot spots (32%), compared with 34 cases with less than 20% positivity of tumor cells' hot spots (68%).

In addition, we observed no statistically significant correlations between AR and Ki 67 activity. A negative association between AR expression and low Ki 67 was reported by Abdlazem et al., as AR positive tumors showed lower Ki 67 expression . This is probably attributed to the anti-proliferative effect of AR.[45]

Recommendations

In order to ensure reliable and reproducible results, we might suggest using a standard cut-off value to define AR immunopositivity.

These biomarkers are recommended to be studied independently and in combination to substantially predict the response to therapy and to detect alternative tumor's strategies that are unlikely to respond to standard therapy.

Future studies are needed with larger number of cases and an adequate follow-up period to confirm the present results and determine the possible responses to anti-androgen endocrine therapy.

Evaluation of the impact of AR status on patients' outcome should be carried out in further studies with larger study samples.

Conclusion

AR-positive TNBC might be a subtype of breast cancer with unique characteristics that may be ideal for antiandrogen endocrine therapy.

EGFR and Endoglin have distinct expression in TNBC indicated that they may be unique biomarkers for targeted therapy and prognosis.

The present study highlighted the benefits of adding AR, EGFR, and CD105 (Endoglin) to the existing marker panel of TNBC.

Acknowledgements

Conflicts of Interest

None declared.

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