



Expression of Androgen Receptor in Invasive Triple Negative Breast Carcinoma

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Introduction

Breast cancer is the most common female cancer worldwide representing nearly a quarter (25%) of all cancers. In India although age adjusted incident rate of breast cancer is lower (25.8 per 100000) than U.K(95 per 100000) but mortality is at par(12.7 vs 17.1 per 100000) with U.K. There is significant increase incidence and cancer associated morbidity & mortality in Indian subcontinent. The age adjusted Incidence rate of Carcinoma of breast was found as high as 41 per 100000 women for Delhi , followed by Chennai (37.9), Bangalore (34.4) & Thiruvananthapuram district (33.7).¹

Breast cancer is the most prevalent and lethal malignancy among females worldwide ². In 2018, 1,735,350 incident breast cancer cases are estimated to be diagnosed in the United States of America and 609,640 associated mortalities are anticipated³. Incidence of TNBC is 15% among breast cancers.

Over the last few decades there have been outstanding advances in breast carcinoma management leading to earlier detection of disease

& development of more effective treatment. Breast cancer is no longer seen as a single disease but rather a multifaceted disease comprised of distinct biological subtype with diverse natural history presenting a varied spectrum of clinical, pathological & molecular features with different prognostic & therapeutic implication.

Invasive breast carcinoma effects younger age women, African- american women and women with BRAC-1 mutation. Invasive breast carcinomas are aggressive and invade the surrounding breast tissue. The prognosis for invasive ductal Carcinoma (IDC) depends on the type of breast cancer, tumor Size, spread, the histological grade and hormonal receptors.

Approximately 80% of breast carcinomas are invasive ductal carcinoma, followed by invasive lobular carcinomas which account for approximately 10-15% of cases.

In Invasive breast carcinoma malignant cells infiltrate and breach the ductal lining and lobules and enter into breast connective tissue, they have the potential to spread to lymph nodes and other organs as metastasis.⁴

Traditionally breast carcinoma is treated with surgery, chemotherapy, radiation, hormonal therapy and targeted therapy. Breast has hormonal receptors namely ER, PR and HER2NEU receptors showing variable expression. About 80% of breast cancers are ER positive that means cancer cells grow in response to estrogen. About 65% of breast cancers are PR positive that means cancer cells grow in response to progesterone. Tumours that are ER and PR positive respond to hormonal/endocrine therapy after surgery and chemotherapy are finished. These hormonal therapies are of two types one by blocking ER receptor [tamoxifen] and second one by decreasing the production of hormone [aromatase inhibitors]. About 20% of breast cancer cells show HER2NEU receptor expression, they respond to targeted therapy with Transtuzumab after surgery and chemotherapy.

HER2 is a gene in breast, that makes HER2 proteins. These proteins are receptors on breast cells that help in growth of normal breast tissue. But in 25% of cases HER2 gene doesn't work and causes HER2 gene amplification, which in turn causes production of excess HER2 proteins. This HER2 gene amplification or overexpression is reported as HER2 positive in pathology reports.⁵ If more than 10% of cells are HER2 positive the breast cancer is HER2 positive. HER2 can be determined by IHC and FISH.

On IHC results can be interpreted as: 0[negative], 1+[also negative], 2+[borderline], 3+[positive-HER2 protein overexpression].

On FISH results are interpreted as: positive [HER2 gene amplification] or negative [no HER2 gene amplification]

HER2 positivity was considered when there was IHC positivity of 3+ [uniform intense complete membrane staining] or a FISH result demonstrating more than 6 copies of HER2 genes per nucleus or FISH ratio of more than 2.2.

HER2 negative result was considered when IHC staining of 0 or 1+ and a FISH result with less than 4 HER2 gene copies per nucleus or a FISH ratio of less than 1.

Based on IHC profile breast carcinomas are classified into 4 groups:

- 1) ER/PR+, HER2NEU+
- 2) ER/PR+, HER2NEU-
- 3) ER/PR-, HER2NEU+
- 4) ER/PR-, HER2NEU- (TRIPLE NEGATIVE BREAST CANCER TNBC)

The IHC classification correlates well with intrinsic gene expression microarray categorization:

- 1) ER/PR+, HER2NEU+ with luminal B
- 2) ER/PR+, HER2NEU- with luminal A
- 3) ER/PR-, HER2NEU+ with HER2NEU over expression
- 4) ER/PR-, HER2NEU- with basal like⁷

TNBCs are invasive ductal carcinomas in which there is loss of expression of ER, PR and HER2NEU, accounts for 15% of breast cancer cases⁸. These cancers have worst prognosis, aggressive and low overall survival and low disease free survival period. TNBC lacks the expression of ER, PR and Her2neu receptors hence they need attention.

Histologically TNBCs are mostly invasive carcinomas which are poorly differentiated and high proliferative activity and large primary tumor showing pushing borders, central necrosis and variable degree of lymphocytic infiltrations. These invasive carcinomas are common in young premenopausal women and those arising from BRCA 1 mutations. Patients with TNBC do not benefit from hormonal or transtuzumab-based therapy because of the loss of target receptors such as ER, PR, and HER-2. Hence, surgery and chemotherapy, individually or in combination, appear to be the only available modalities. Treatment of patients with these phenotypes has been challenging because of aggressive behavior. Approximately 10-32% of TNBC showed androgen receptor signaling in such patients AR blockade would be a potential endocrine treatment.

Androgen receptor is a member of nuclear steroid hormone receptor family, which also include ER

and PR. Steroid hormone receptor are critical component of signaling pathway and play a crucial role in transcription factors regulating gene expression. Although ER and PR are widely recognized for their prognostic and predictive role in breast cancer, the biological role of AR in breast carcinoma is emerging. The drug development pipeline of AR targeted therapies in prostate cancer is facilitating the evaluation of AR signal inhibition in TNBC including bicalutamide [a non-steroid partial agonist] and enzalutamide [an inhibitor of nuclear localization of AR].⁹

Expression of EGFR is seen in 15-45% of breast cancer. Its expression was mainly found in basal-like carcinoma .EGFR activation results in cell proliferation, angiogenesis, invasion and metastasis. A majority of triple negative patients have tumors of basal subtype with EGFR expression and poor prognosis.

Materials and Methodology

Study Design: a crosssectional analytical diagnostic study of MRM specimen sampled for routine histopathology at department of pathology yashoda hospital secunderabad.

Sample Size: minimum of 50 cases of breast cancer cases whose IHC interpretation was ER/PR/HER2NEU negative as per ASCO / CAP guidelines.

Sample size was calculated by using simple formula (Daniel 1999):

$$n = \frac{Z^2 P(1-P)}{d^2}$$

where n = sample size,

Z = Z statistic for a level of confidence, for the level of confidence of 95%, which is conventional Z value is 1.96.

P = expected prevalence or proportion (in proportion of one; if 15%, P = 0.15)

d = precision (in proportion of one; if 10%, d = 0.10).

Study Duration: 2 year study period between July 2017 to June 2019.

Method: A systematic study of Crosssectional study of total 50 patients who underwent surgical resection for breast carcinoma at yashoda hospitals from 2017 to 2019 were enrolled in the study.

A single tumor block of paraffin embedded tissue from resected specimens was selected. For construction of tissue microarrays [TMA] the most representative area of tumor was identified on H and E slide and marked. A replicate core sample measuring 0.6mm in diameter at a spacing of 0.7-0.8mm were obtained using a precise instrument, and arrayed on a recipient paraffin block. Using microtomy 4micron thickness sections were cut from TMA blocks and stained for H and E to verify the histology. Here we have taken 20 cases on one TMA slide.

For IHC the panel of markers taken were ER, PR, HER2NEU, AR and EGFR.

Procedure

- 1] 4micron thickness sections were cut from TMA blocks and attached on polylysine coated slides and incubated at 37 degrees Celsius for two hours.
- 2] The slides were dewaxed in xyeline and rehydrated in graded alcohols and covered with citrate buffer [pH -6] for 15 minutes.
- 3] Antigen retrieval was done in microwave at 600 watt for 15 minutes.
- 4] Cooled to room temperature and washed with tap water.
- 5] Peroxidase block was done for 5 minutes.
- 6] Incubated for 1hour with primary monoclonal antibodies against ER, PR, HER2NEU, AR and EGFR.
- 7] Washed with phosphate buffer saline 2-3 times.
- 8] Polyexcel target binder was added for 15 min.
- 9] Again washed with phosphate buffer saline 2-3 times.
- 10] This was followed by incubation with secondary antibody for 15minutes
- 11] Washed with PBS 2-3 times
- 12] DAB (diaminobenzidine) was added for 5minutes.
- 13] Washed under tap water for 5 minutes.

- 14] Counterstained with hematoxylin for 30seconds.
- 15] Water washed.

- 16] Dehydrated with xylene and alcohol.
- 17] Dry the slide and mount with DPX (dextreneplastisizer of xylene)

Table-2 list of primary antibodies

| | ER | PR | HER2NEU | AR | EGFR |
|------------------|------------|------------|------------|------------|------------|
| CLONE | EP1 | EP2 | EP3 | EP120 | EP22 |
| SPECIES | Rabbit | Rabbit | Rabbit | Rabbit | Rabbit |
| DILUTION | 1:100 | 1:100 | 1:200 | RTU | RTU |
| CATALOGUE NUMBER | CR042 | CR068 | CR047 | PR002 | PR040 |
| CLONALITY | Monoclonal | Monoclonal | Monoclonal | Monoclonal | Monoclonal |
| SOURCE | pathnsitu | Pathnsitu | pathnsitu | Pathnsitu | Pathnsitu |

(RTU-ready to use)

Then the sections were assessed for ER, PR and HER2NEU status. ER and PR were assessed based on guidelines provided by Allered method. For ER and PR negative (0% cells positive) was included in study. For HER2NEU ASCO/CAP guidelines were followed, score 0 was taken negative in HER2NEU.

Clinical details like age of the patient, tumor size, grade and lymph node status were collected from case records. The study was conducted after obtaining approval from ethics committee and informed consent from patient.

Statistical Analysis

The data analysis was done by using SPSS (statistical package for social sciences) windows version 24.0 for statistical analysis.

Qualitative data variables were expressed by frequency / percentages.

Quantitative data variables were expressed by using mean and standard deviation.

To study the association between AR and MIB, age, tumor size, grade, lymph node status and EGFR by chi square test.

Risk estimate with 95% CI was calculated by odds ratio.

Level of significance was considered as 0.05.

Age

Table-3 frequency distribution of age

| | N | Minimum | maximum | Mean | Std. deviation |
|---------|----|---------|---------|-------|----------------|
| Age | 56 | 30 | 75 | 52.52 | 11.989 |
| Valid N | 56 | | | | |

Results

56 women aged between 30-75 yrs who were diagnosed with TNBC were assessed for androgen receptor (AR) expression. Among 56 cases 2 were medullary carcinoma,1 was solid papillary carcinoma and remaining 53 were invasive ductal carcinoma. All the patients underwent mastectomy or lumpectomy as primary treatment. Incidence of TNBC patients at our hospital was 6.5%.

Mean age of TNBC diagnosis was 52yrs. In TNBC cases predominantly tumor size was pT2 (82%), grade was G3 (73%) and lymph node status was N0 (58.9%).

AR and EGFR were interpreted by immunohistochemistry. For AR positivity nuclear staining and for EGFR positivity membranous and cytoplasmic staining was considered. 10% cut off was taken for AR positivity and 15% for EGFR. On analysis AR was positive in 21.4% of cases and EGFR was positive in 58.9% of cases.

Tumor Size (pT)

Table -4 frequency distribution of tumor size

| | Frequency | Percent | Valid percent | Cumulative percent |
|----------|-----------|---------|---------------|--------------------|
| Valid T2 | 46 | 82.1 | 82.1 | 82.1 |
| T3 | 10 | 17.9 | 17.9 | 100.0 |
| Total | 56 | 100.0 | 100.0 | |

Grade (G)

Table-5 frequency distribution of tumor grade

| | Frequency | Percent | Valid percent | Cumulative percent |
|----------|-----------|---------|---------------|--------------------|
| Valid G2 | 15 | 26.8 | 26.8 | 26.8 |
| G3 | 41 | 73.2 | 73.2 | 100.0 |
| Total | 56 | 100.0 | 100.0 | |

Lymph Node (N)

Table-6 frequency distribution of lymph node metastasis

| | Frequency | Percent | Valid percent | Cumulative percent |
|----------|-----------|---------|---------------|--------------------|
| Valid N0 | 33 | 58.9 | 58.9 | 58.9 |
| N1 | 9 | 16.1 | 16.1 | 75.0 |
| N2 | 8 | 14.3 | 14.3 | 89.3 |
| N3 | 6 | 10.7 | 10.7 | 100.0 |
| Total | 56 | 100.0 | 100.0 | |

Androgen Receptor (AR)

Table-7 Frequency distribution of androgen receptor

| | Frequency | Percent | Valid percent | Cumulative percent |
|----------------|-----------|---------|---------------|--------------------|
| Valid negative | 44 | 78.6 | 78.6 | 78.6 |
| Positive | 12 | 21.4 | 21.4 | 100.0 |
| total | 56 | 100.0 | 100.0 | |

EGFR

Table-8 Frequency distribution of EGFR

| | Frequency | Percent | Valid percent | Cumulative percent |
|----------------|-----------|---------|---------------|--------------------|
| Valid negative | 23 | 41.1 | 41.1 | 41.1 |
| Positive | 33 | 58.9 | 58.9 | 100.0 |
| Total | 56 | 100.0 | 100.0 | |

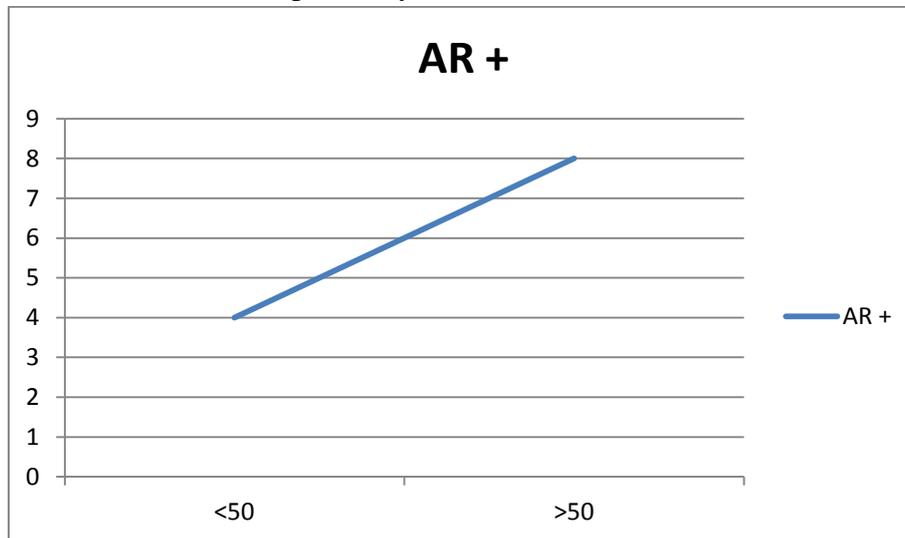
Table-9: Clinico -pathological correlation of AR positive and AR negative TNBC:

| | Total | AR positive | AR negative | Wilcoxon test P-value |
|------------|-------|-------------|-------------|-----------------------|
| AGE | | | | |
| <50yrs | 23 | 4 | 19 | |
| >50yrs | 33 | 8 | 25 | 0.393 |
| TUMOR SIZE | | | | |
| T2 | 46 | 11 | 35 | |
| T3 | 10 | 1 | 9 | 0.309 |
| GRADE | | | | |
| G2 | 15 | 5 | 10 | |
| G3 | 41 | 7 | 34 | 0.171 |
| LYMPH NODE | | | | |
| N0 | 33 | 5 | 28 | |
| N1 | 9 | 3 | 6 | |
| N2 | 8 | 3 | 5 | |
| N3 | 6 | 1 | 5 | 0.445 |
| MIB% | | | | |
| <14% | 25 | 9 | 16 | |
| >14% | 31 | 3 | 28 | 0.019 |
| EGFR | | | | |
| <15% | 23 | 3 | 20 | |
| >15% | 33 | 9 | 24 | |

Prevalence: 10% was taken as threshold to define AR positivity. 26.7% (15/56) of TNBC were AR positive, of which 21.4% (12/56) were AR positive with >10% of cells and 5.3% (3/56) cases were AR positive with <10% of cells.

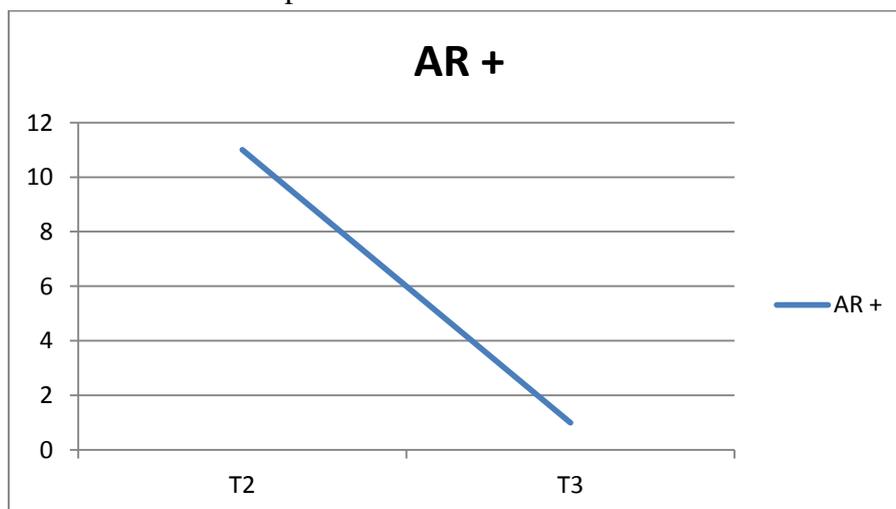
Age: mean age of TNBC patients was 52.52 yrs. AR was predominantly positive in older women ($p=0.393$)

Figure-15 Age wise distribution of AR positivity



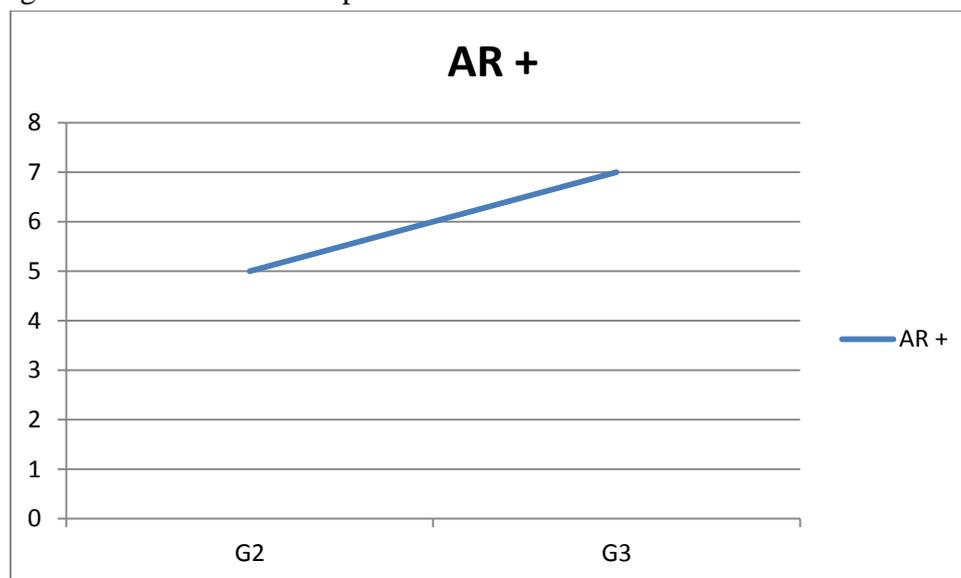
Tumor size: in AR positive TNBC patients tumor size was predominantly T2 ($p= 0.309$)

Figure-16 tumor size distribution in AR positive cases



Grade: in AR positive TNBC patients, 7 patients were grade 3 and 5 patients were grade 2, indicating higher grade predominance in AR positive patients. ($p=0.171$). Overall TNBC have higher grade.

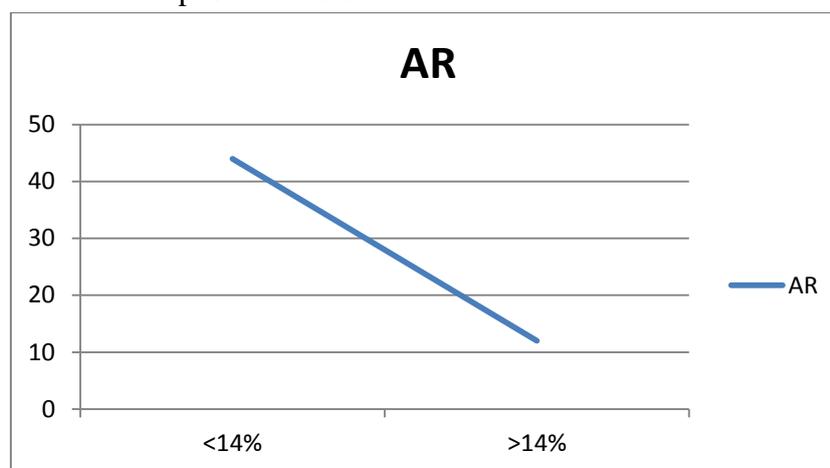
Figure-17 tumor grade distribution in AR positive cases



Lymph node: in AR positive patients 5 patients were N0, 3 patients were N1, 3 patients were N2 and 1 patient was N3 (p= 0.445). There wasn't any significant association between AR positivity and lymph node metastasis.

MIB%: there was an inverse relation between AR positivity and MIB%. Lesser the MIB% more the AR positivity (p=0.019)

Figure-18 MIB distribution in AR positive cases



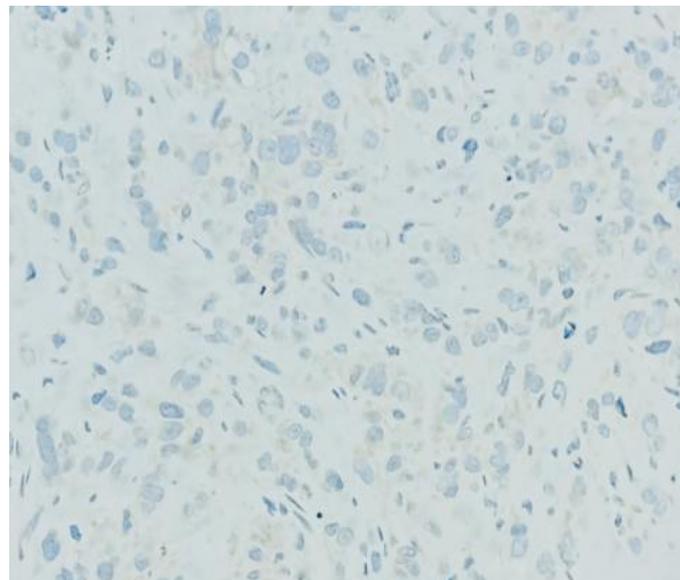
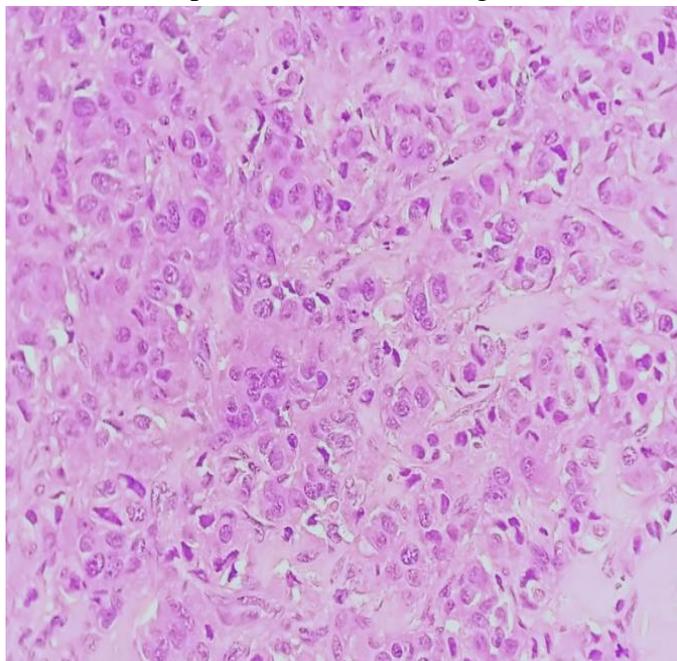
EGFR: 15% cut off was taken for EGFR positivity, almost all the TNBCs expressed basal marker EGFR. Among 12 AR positive cases, 9 patients were EGFR positive, and 3 patients were EGFR negative (p= 0.173). Though 25% patients did not show basal phenotype but 75% of patients were AR+, which is a significant value and cannot be ignored, these patients can be considered for targeted therapy. 3 cases which were medullary and papillary phenotype on histology were negative for EGFR expression indicating there

low grade nature and good prognosis. Though these cases are triple negative but not basal like. To summarize the results, of all the parameters AR showed significant association with MIB%. AR association with other parameters showed as its positivity is more in elderly women, higher grade, lower tumor size and lower lymph node status. Other parameters did not get significant value because of low sample size and poor patient compliance in a tertiary health care center.

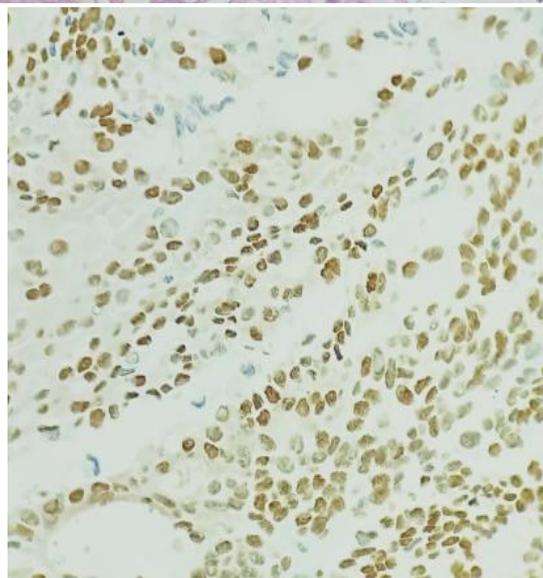
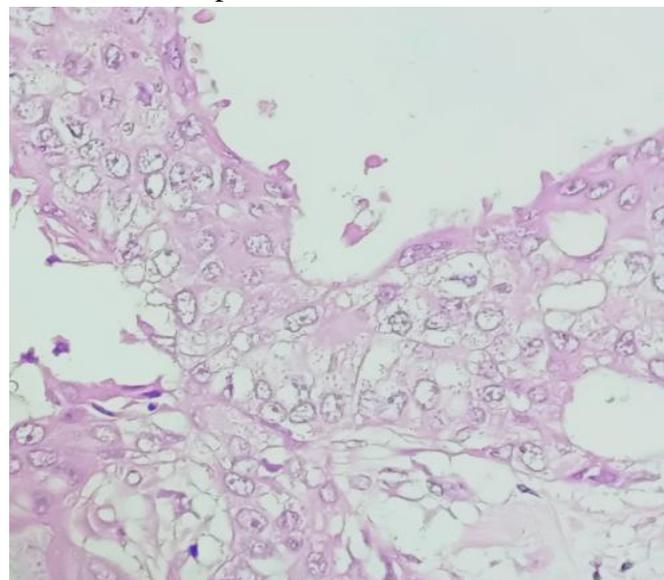
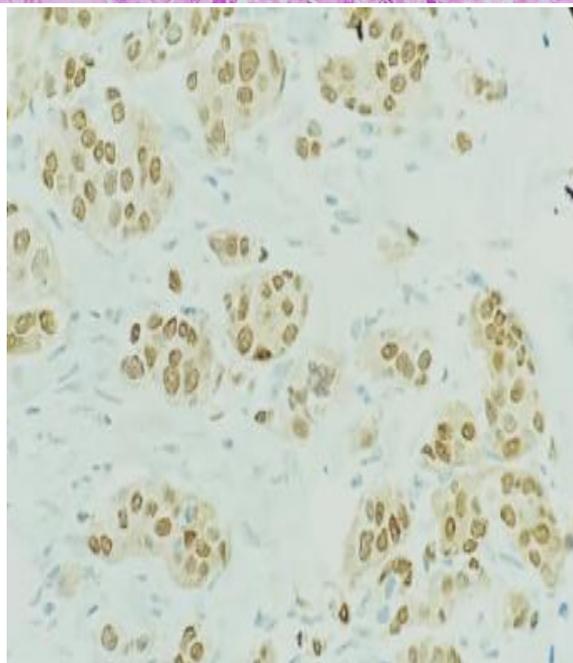
EGFR did not reveal any significant association with any of the parameters.

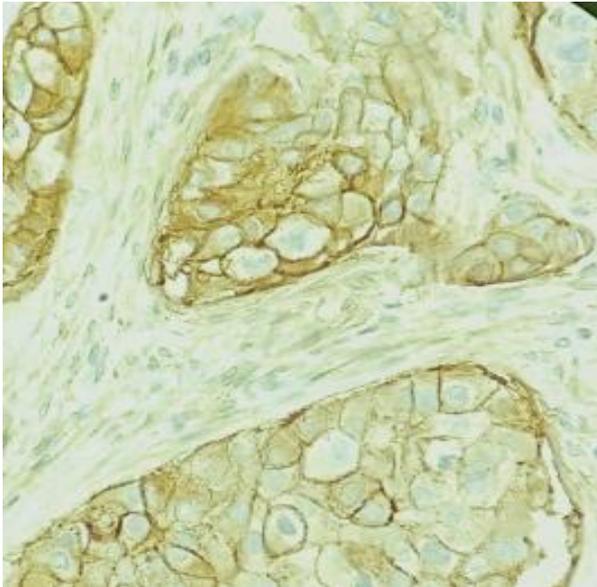
Microscopic images

Figure-20: 40 year old female showing high grade IDC, with AR positive and EGFR negative

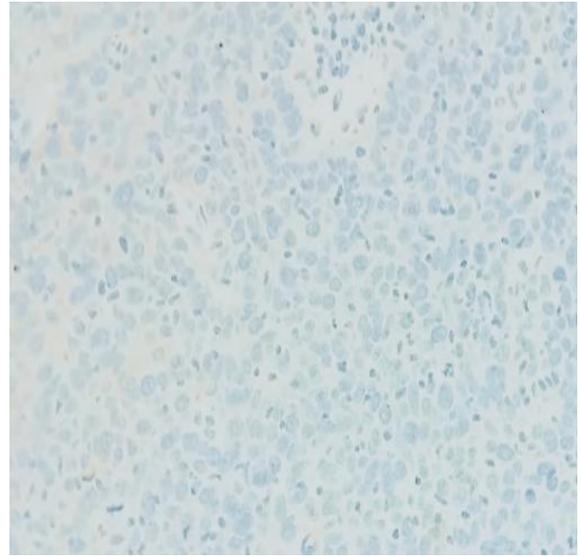
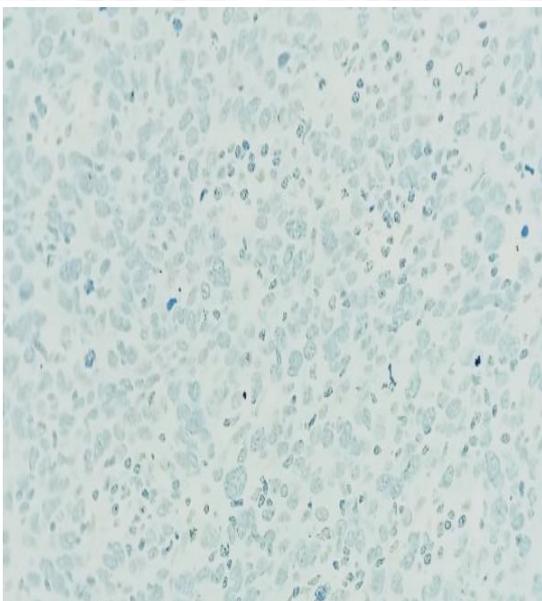
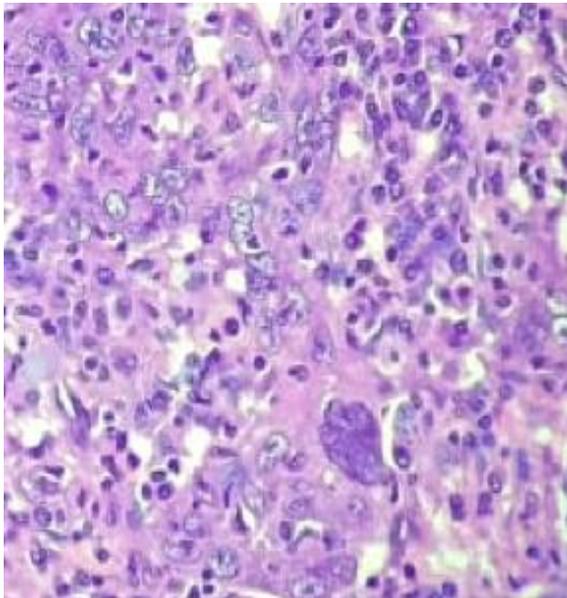


55 year old female with high grade IDC showing AR and EGFR positive

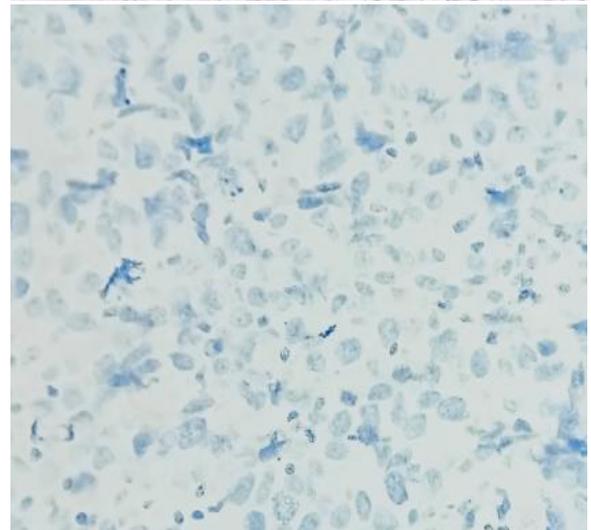
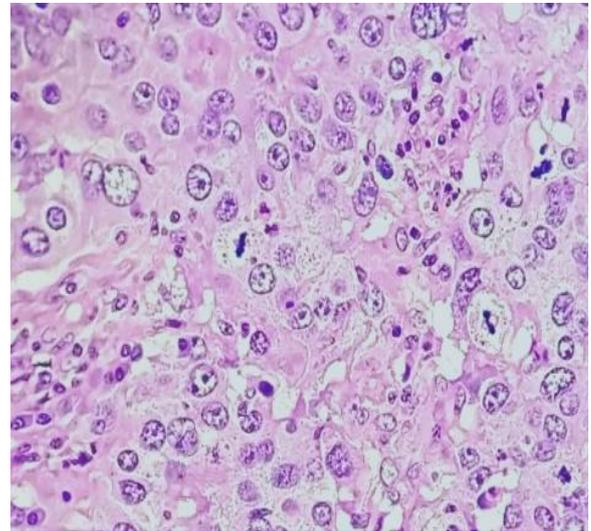


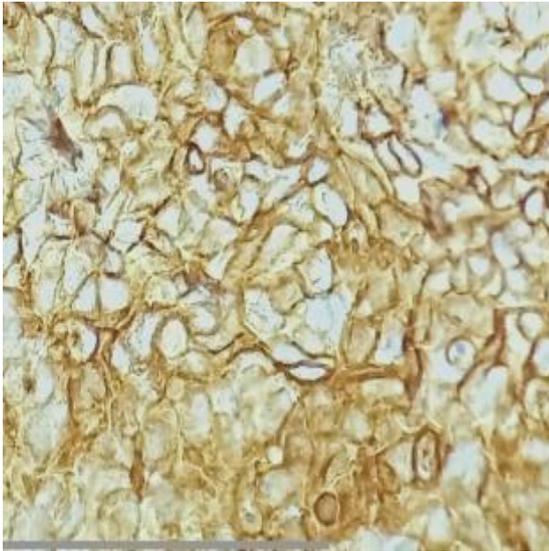


30 years old female with IDC medullary type showing AR and EGFR negative

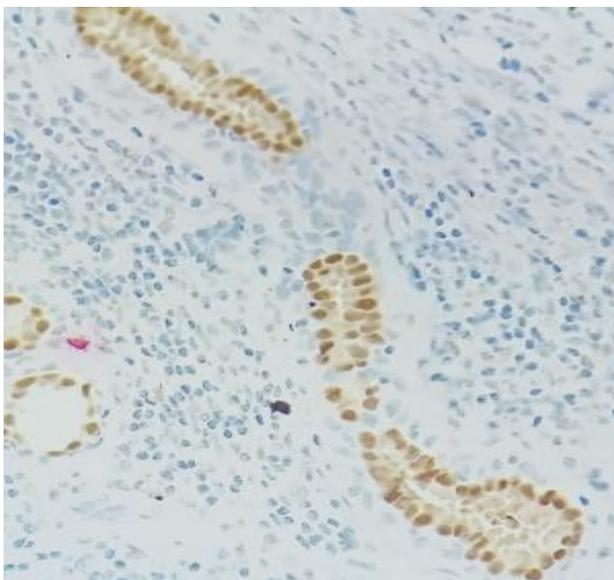


68 year old female with high grade IDC, showing AR negative and EGFR positive





AR positivity seen in normal breast ductal elements



Gross Images

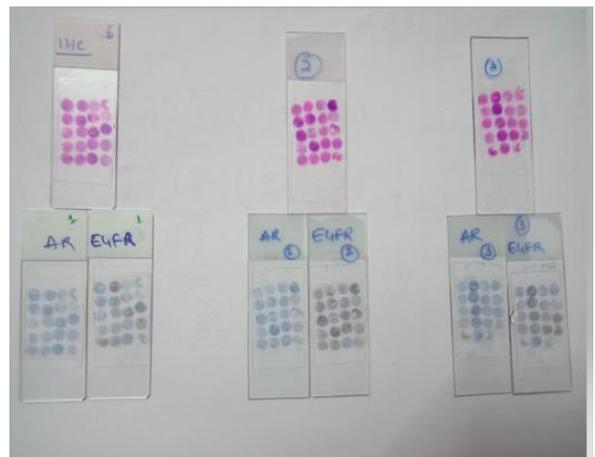
Right modified radical mastectomy



Right modified radical mastectomy



TMA Blocks and Slides



Discussion

Androgen receptor is normally present in nuclei of mammary gland tissue epithelial cells. Studies have demonstrated AR is seen in nuclei of ductal and alveolar cells by immunohistochemistry¹⁰⁻¹⁴.

Few studies have showed that androgens oppose estrogens in the mammary gland. Studies conducted on rhesus monkeys demonstrated the inhibiting influence of androgens on mammary epithelial proliferation by reducing estrogen induced proliferation^{15,16}. Similar results were seen in rodent mammary gland¹⁷.

The effects of androgens on breast cancer cell lines depends on the presence of the AR, the concentration of androgen and type of androgen used along with the presence of co-regulatory proteins¹⁸. In addition, apoptotic effects of androgens on breast cancer cell lines have been demonstrated¹⁹. Similar findings have been seen in cancer cells of the prostate²⁰.

Androgen receptor plays a key role in mammary gland development like ductal branching, formation of milk producing alveoli and lobuloalveolar development²¹. There are few clinical evidences indicating role of AR in cancer progression²²⁻²⁴. In present study, the expression of AR in 56 TNBC patients was determined using immunohistochemistry.

Our data revealed 21.4% of 56 TNBC cases are positive for AR expression. This was consistent with previous findings that 10-53% of TNBC tumors express AR²⁵⁻²⁷. In present study all TNBC cases were diagnosed following the most recent guidelines recommended for evaluation of ER, PR, HER2NEU, AR and EGFR²⁸⁻²⁹.

These results of present study showed that AR is detected more frequently in smaller tumors, in cases with no lymph node metastasis, higher histologic grade and with low MIB.

Basal like breast carcinomas generally characterized by lack of hormone receptor expression and show positive expression for basal epithelial cell markers. EGFR marker which can be overexpressed in basal like carcinoma of breast included in our study, it was expressed in 58.9% of all triple negative cases. EGFR is expressed in high grade tumours and the tumors have tendency to metastasis. Thus it is useful clue to predict the tumour nature. EGFR was expressed in 75% of AR positive cases, indicating significant

association. Though EGFR targeted therapy trials was conducted but the results was not satisfactory. One of the hypothesis states that the chemotherapy drugs acts more on AR- cells than AR+ cells, resulting in the up regulation of AR gene expression and AR+ cells exhibiting chemotherapeutics resistance³⁰, thus AR hormone receptor negative breast cancers have more chances to benefit from chemotherapy. Chemotherapy insensitive or resistant triple-negative breast cancer may have high levels of AR expression; that's why, AR-targeted therapy may be used in AR+ TNBC, which poorly responds to chemotherapy.

In ER-negative TNBC, AR provokes tumor growth by stimulating the ER signaling pathway⁸⁷. As in molecular apocrine profile (ER-, AR+), it shows a high invasive capability and poor prognosis³¹. 90% of patients with TNBC have genetic alterations like mutations, deletions or amplifications³².

Prostate cancer is the second most common cancer among males in worldwide and it is also a hormone dependent cancer³³, and modifying the androgen levels can be useful in the treatment of prostatic carcinoma³⁴. Therefore, AR targeted therapy can be productive in a particular group of patients, those with AR+ TNBC may have increase chances of survival rates. By using bicalutamide in treatment of prostate carcinoma archived great results³⁵, numerous preclinical or clinical studies were conducted for using AR antagonists in TNBC (53-57). LAR breast cancer cell lines are sensitive to AR antagonists. On Further, study Cuenca-Lopez et al³⁶ reported that AR+ TNBC cell line, those which do not belong to the LAR subtype, also are sensitivity to AR antagonists. In early clinical trials, patients with advanced AR+ TNBC were treated with bicalutamide with a clinical benefit rate of 20%³⁷. In a phase II clinical trial of enzalutamide, which has a six-fold higher affinity to AR than bicalutamide, 42% of patients with advanced AR+ TNBC achieved a clinical benefit time of 16 weeks in the initial data³⁸.

Selective androgen receptor modulators (SARMs) are novel AR-targeted therapies, which have high specificity for AR without masculinizing side effects. Additionally, SARMs improve the side effects of advanced breast cancer by increasing muscle mass and restoring bone mineral density³⁹. The study of Lehmann et al⁴⁰, discovered that in AR+ TNBC cells, PI3K/mTOR inhibitors in combination with an AR antagonist had an additional growth inhibitory effect.

In TNBC tumors, it was seen that positivity by AR immunostaining is a favorable prognostic factor and was associated with a lower clinical stage, higher histologic grade, and lower proliferation index.

Absence of AR expression is associated with an increased risk for recurrence and distant metastasis in lymph node-positive TNBCs^{41,42}.

Similarly, Luo and colleagues have shown AR expression to be correlated with higher 5-year disease-free survival (DFS) and overall survival (OS) in patients with TNBC⁴³.

Routine AR evaluation in TNBC by IHC will provide further awareness in this path.

Newer treatment strategies

In addition to single-agent studies, significant interest is being shown in potential strategies combining AR antagonists with other targeted treatments. Lehmann and colleagues have found AR-positive TNBC tumors to have a higher frequency of phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha gene (PIK3CA) mutations compared with AR-negative tumors (40% vs 4%), and these are often associated with concurrent amplification of the PIK3CA locus. In cell line models and xenograft studies, combining bicalutamide with a pan-phosphoinositide 3-kinase (PI3K) inhibitor or a dual PI3K/mammalian target of rapamycin (mTOR) inhibitor has shown additive effects. This has resulted in studies assessing combinations of AR-targeted therapies with PI3K/mTOR inhibitors in advanced AR-positive TNBC tumors⁴⁴. A phase 1b/2 clinical trial of taselisib, a PI3K inhibitor, in combination with enzalutamide

in advanced TNBC is currently recruiting patients (NCT02457910)⁴⁵. Cyclin-dependent kinases 4 and 6 (CDK4 and CDK6), activated by cyclin D, promote cell cycle entry by phosphorylating Rb (retinoblastoma) and other proteins to initiate transition from the G1 phase to the S phase⁴⁶.

This pathway is disrupted in many human cancers, leading to unrestrained cell proliferation⁴⁷. After showing encouraging preclinical efficacy in breast cancer, several inhibitors of CDK4 and CDK6 are currently in varying stages of development⁴⁸. Based on the significant improvement seen in PFS, the CDK4/6 inhibitor palbociclib (Ibrance, Pfizer) has been approved for the frontline treatment of advanced ER-positive breast cancer in combination with letrozole⁴⁹. Recent data also support the role of palbociclib with fulvestrant (Faslodex, AstraZeneca) in patients who have had prior progression on endocrine therapy⁵⁰. Although TNBC has an inherently higher proliferation rate, limited activity has been seen in preclinical studies with CDK4/6 inhibitors in unselected TNBC tumors. However, more recently, the LAR subtype of TNBC has been shown to be particularly susceptible to CDK4/6 inhibition⁵¹.

Conclusion

Anti-AR therapy has been a significant advancement in the treatment of TNBC tumors considering the fact that lack of targeted therapy for TNBC. AR represents a novel targeted therapy in TNBC, which has an otherwise poor prognosis. Based on the reliable clinical data and immunohistochemistry results, we expect that the newer, more potent anti-androgens will significantly improve the outcomes and make the targeted therapy available for what to date has been an orphan disease.

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