



Original Research Article

Immunohistochemical expression of ER, PR, HER2, Ki67, CK5/6 and BRCA1 in non familial breast cancer and its correlation with clinico-pathological parameters

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ABSTRACT

Background: Triple negative breast cancers (TNBC), a pathologically high grade subset of tumours has increasing prevalence in Indian population. Since their morphological features resemble with BRCA1 mutated inherited breast cancer, the present study was aimed to define the role of BRCA1 mutation in sporadic breast cancer.

Aims and Objectives: 1. To evaluate immunohistochemical expression of BRCA-1 in non familial breast cancer cases and to correlate with clinical and histopathological parameters.

Setting and Design: Prospective & Retrospective study (August 2014-August 2015).

Tertiary care hospital in North Eastern India

Materials and Methods: 189 cases of suspected breast cancer cases were included in the study. ER/PR/HER2 status was evaluated of 114 cases and molecular classification along with BRCA1 expression was seen in 62 patients.

Statistical analysis: SPSS (Statistical Package for Social Sciences) Version 15.0 statistical Analysis Software. The values were represented in Number (%) and Mean±SD.

Result: 71.56% of cases belonged to Nottingham's grade II and III. Among our groups maximum percentage were of TNBC (basal + non basal) and Her2 enriched i.e (30.6% respectively) followed by luminal B (24.2%) and luminal A (9.6%). 50% of cases in study sub population showed BRCA 1 loss. BRCA1 expression was nuclear and cytoplasmic in both control and cases. Maximum mean BRCA1 loss was seen in basal like, followed by triple negative non basal like, her2 enriched and minimum in luminal A.

Conclusion: Large percentage of TNBC, increased BRCA1 expression loss and high grade, indicates need to fulfil the vacant space of therapeutic modality in TNBC management. PARP inhibitors, the future of therapeutic management of TNBC might also be helpful in sporadic cases of breast cancer exhibiting BRCA1 expression loss.

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1. Introduction

Carcinoma breast is emerging as one of the leading cause of cancer related deaths in India. The recent ICMR data states that breast cancer contribution to total cancer burden

in India accounts for 27.3% outnumbering cancer cervix and ovary which accounts for 14% of the total cases.¹ Even more worrisome in the present scenario is the shift seen in breast cancer with new victims being females in younger and middle age group and also with an unfavourable morphological and hormonal profile.²

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Hormonal therapy is preferred treatment of choice in Estrogen Receptor (ER) and Progesterone Receptor (PR) positive breast cancer and trastuzumab (herceptin) in her2/neu positive breast cancer.³

Still a subset of patients are left out which do not express the above and remain deprived of the added benefits of hormonal regimens. Moreover such cases are pathologically of high grade, with limited treatment options and hence represent a poor clinical outcome compared with the hormone therapy responders.⁴

After the worldwide establishment of hormonal therapy for carcinoma breast there has been a constant hunt for novel therapeutic options which would address the above mentioned high grade and hormone non responding group i.e triple negative breast cancer (TNBC) more recently molecular subtype as basal-like. Apart from the fact that such tumours are triple negative they exhibit certain morphological and molecular features such as high grade with high mitotic count, pushing margins and syncytial growth, confluent necrosis, basal like gene expression profile of cytokeratins 5/6, 14, and 17, trabecular growth pattern and frequent TP53 mutation.⁵

Since these morphological features are associated with BRCA1 mutated inherited breast cancer, studies aimed to define the role of BRCA1 mutation in sporadic breast cancer were designed.⁶

The assessment of BRCA status is of importance not only for grading of tumour, but also in adding therapeutic and prognostic dimensions. There is limited data in literature exploring its status in Indian population. So the present study was designed to study the breast carcinoma cases, evaluate their hormonal status in terms of molecular classification based on Immuno-histochemical expression of ER/ PR/ Her2neu/ CK5/6 and Ki67. Also assess the Immunohistochemical expression of BRCA1 in breast cancer specimens received in study duration irrespective of hormone status/ treatment status or absence or presence of family history and to co-relate them with the morphology and hormonal status.

2. Review of Literature

The BRCA1 gene is located on the long arm of chromosome 17 at position 21 and belongs to a class of tumour suppressor genes. BRCA1 protein has many functions, including homologous recombination DNA repair (HRR), cell cycle check point control, ubiquitylation, chromatin remodelling, and DNA decatenation. Breast cancer 1 early onset classified as low frequency high penetrance breast cancer predisposition genes is associated with cancer breast and ovary. This gene is involved in DNA repair. Deficiency in homology directed DNA repair causes high level of genomic instability and increased risk of tumorigenesis.³ In normal cells, the HRR pathway is activated in response to DNA double-stranded break. In BRCA 1/2deficient cells, HRR is

faulty secondary to loss of BRCA function, and therefore, other more error-prone DNA repair pathways are activated. These less perfect mechanisms lead to tumorigenesis.³ Alternatively this depleted repair machinery can be used as blessing in disguise to model novel therapeutic strategies which even block the alternate repair mechanism of cell and in the background of BRCA depleted cells forcing the tumour cells to apoptosis. The model enrolls PARP 1 inhibitor i.e. Poly adenosine di-phosphate (ADP) –ribose polymerase-1 (PARP 1) inhibitors and platinum based chemotherapy.^{7,8}

3. Materials and Methods

A prospective & Retrospective study was done from August 2014-August 2015 at a tertiary care hospital in North Eastern India. Modified radical mastectomy/lumpectomy/biopsy specimens, received in the department of pathology during the study duration were included in the study. Using a clinical case sheet consent of patient, age, chief complaints, family history of breast cancer in maternal two generation, associated ailments, obstetric history and lactational history was recorded. In case of MRM/lumpectomy/BCS a previous record of neo-adjuvant chemotherapy was taken.

Follow up data of the patients was retrieved by telephone calls to the patients and the current status of the patient was noted (Alive, recurrence or Dead with the time of death).

189 cases of suspected breast cancer cases were received during study duration. Cases of breast cancer other than IDC (NOS) (06), male breast cancer (12), specimens without tumour tissue(12), Inadequate/exhausted/unprocessable histo-pathological material for performing I.H.C (23) Familial IDC (NOS) (01), prepared blocks (05), and axillary lymph-node cases with IDC metastases (06) were excluded from the study. Histo-pathological categorization of 124 cases of breast carcinoma was done under CAP protocol and tumour grading under the Nottingham modification of the Bloom–Richardson system. ER/PR/HER2 status was evaluated in 114 cases. Molecular classification with further BRCA1 expression (highlighting somatic BRCA1 mutation) was evaluated in 62 cases. Immunohistochemical evaluation using Streptavidin Biotin immunoperoxidase method was done. Staining and evaluation was done using specific polyclonal antibodies as per Standard protocol. CK 5/6-cytoplasmic moderate to strong more than or equal 1% in invasive tumor cells was taken as positive. The Following clones of primary antibodies were used.

3.1. Primary antibody used

1. BRCA1- polyclonal rabbit, RTU (AR345-5R (BioGenex
2. ER - Flex polyclonal rabbit -a Hu ER alpha, Clone EP1, RTU (DAKO AS/AS+

3. PR- Flex Monoclonal Mo a Hu PR, Clone PgR636, RTU (DAKO AS/AS+
4. HER2 - polyclonal rabbit a Hu c-erb2 oncoProtein, RTU (DAKO AS/AS+
5. Ki67 - Flex Monoclonal Mo a Hu Ki67 Antigen, Clone MIB-1, RTU (DAKO AS/AS+
6. CK5/6 - Flex Monoclonal Mo a Hu ck5/6 protein, Clone D5/16 RTU (DAKO AS/AS+

BRCA1 expression (nuclear & cytoplasmic) was calculated. Fibroadenoma and adjacent normal glands of breast parenchyma was taken as positive control (Figure 3).

Nuclear and cytoplasmic expression in control was assumed as 100% and percentage nuclear loss and cytoplasmic loss of expression was calculated using the following scoring pattern (Figure 2, Table 1)

The statistical analysis was done using SPSS (Statistical Package for Social Sciences) Version 15.0 statistical Analysis Software. The values were represented in Number (%) and Mean±SD.

4. Results

Out of total 189 patients of breast cancer, 124 patients were included in the study.

In the present study, the youngest patient was 26 years old while oldest was 90 years. Median age was 45 years and mean age was 47.98±10.55 years. Majority of our study population was in NG II (65.14%) followed by NGI (28.44%) and III (6.42%) respectively.

Size of tumour in alive cases (101.68±213.47 cm³) was found to be significantly lower than that of cases with recurrence (399.00±429.21 cm³) and patients who died (466.40±236.53 cm³). Correlation between tumor size and outcomewas statistically significant (p=0.006)(Figure 1 LU).

Size of tumour was evaluated in MRM/lumpectomy specimens and was found that 8% of cases had size of <2cm and 92% had tumour size > 2cm.

Distribution of cases (n=114) on the basis of ER/PR/HER2 was as follows (Table 2).

7% of the cases in the study were triple negative and Her2 enriched each respectively.

Proportion of TNBC was low in grade I (22.73%) and was higher in Nottingham Grade II + Grade III i.e 42.5%, however the correlation was not statistically significant (p=0.119). Mean tumor size of TNBC cases was found to be higher than that of non-TNBC cases but this difference was not statistically significant.

Further 62 cases were evaluated for BRCA status, CK5/6 and KI67 status in addition to ER/PR/HER2 profile.

According to molecular classification the distribution of cases (n=62) was as follows - TNBC (Basal) were 03 (4.8%), TNBC(non basal) 17 (27.4%), Luminal A -06 (9.7%), Luminal B- 15 (24.2%) and HER2 enriched were 21

(33.9%). In the study sub population also maximum cases belonged to HER2 enriched and TNBC category.

Proportional differences in outcome of patients of different molecular class were observed. Prognosis wise basal like was worse with a mortality of 50%. This was followed by TNNBL contributing 66.67% of total recurrences and 20% of total mortality and HER2 enriched contributing 40% of total mortality. Luminal A showed best outcome with no mortality/recurrence. However this association was not found to be statistically significant (p=0.737). (Figure 1 RU). Mean KI67 levels of dead cases was found to be higher than that of Alive and cases with recurrence, but association was not statistically significant (p=0.799).

Maximum mean loss of BRCA1 expression (nuclear+ Cytoplasmic) was seen in basal like subgroup (68.06) % followed by triple negative non basal like (51.72)%. Her2 enriched had 48.53% loss. Luminal B had 42.86% while least BRCA1 loss was seen in luminal A (36.81)%, however the correlation was not statistically significant (Figure 1 LL). Mean loss of BRCA1 expression of grade II was maximum (52.86)% followed by grade III 42.86%) while minimum in grade I (31.85%). Maximum BRCA1 loss was seen in demised patients (67.50%) followed by almost equal loss in alive and recurrence cases (42.41%) and (37.50%) respectively. (Figure 1 RL). All the patients of Nottingham Grade I (100.0%) were alive while proportion of patients who died/ recurrence was found only in grade II and grade III i.e 27.27% & 50% respectively.

5. Discussion

The large majority of breast cancers are detected during the reproductive and midlife years. The incidence curve starts rising at puberty, increases steeply up to menopausal age, and levels off afterwards.¹¹ In the present study, maximum number of patients of carcinoma breast belonged to peri-menopausal age group (41-50 yrs). The findings were in agreement with a study by Tiwari S et al. in 2015.¹² Approximately $\frac{1}{4}$ of the cases (24.9%) belonged to young age i.e less than 35 years. This is in concordance with the previous studies which quote that the proportion of young patients varies from about 10% in developed to up to 25% in developing Asian countries including India.¹³

91.7% of cases had tumour size more than 2cm, reinforcing the fact that in the developing countries like India, breast cancer patients are diagnosed at a relatively late stage. This can be attributed to illiteracy, lack of awareness and screening programmes, poor economic infrastructure and low priority in public health schemes.¹³

Maximum of the cases belonged to grade II (65.14%) and Grade I (28.44%) with a small subset (6.42%) belonging to grade III. These findings were in concordance with observation of Bal A et al. (2012) where grade II was maximum (57.1%).⁹ On further analysis we found that

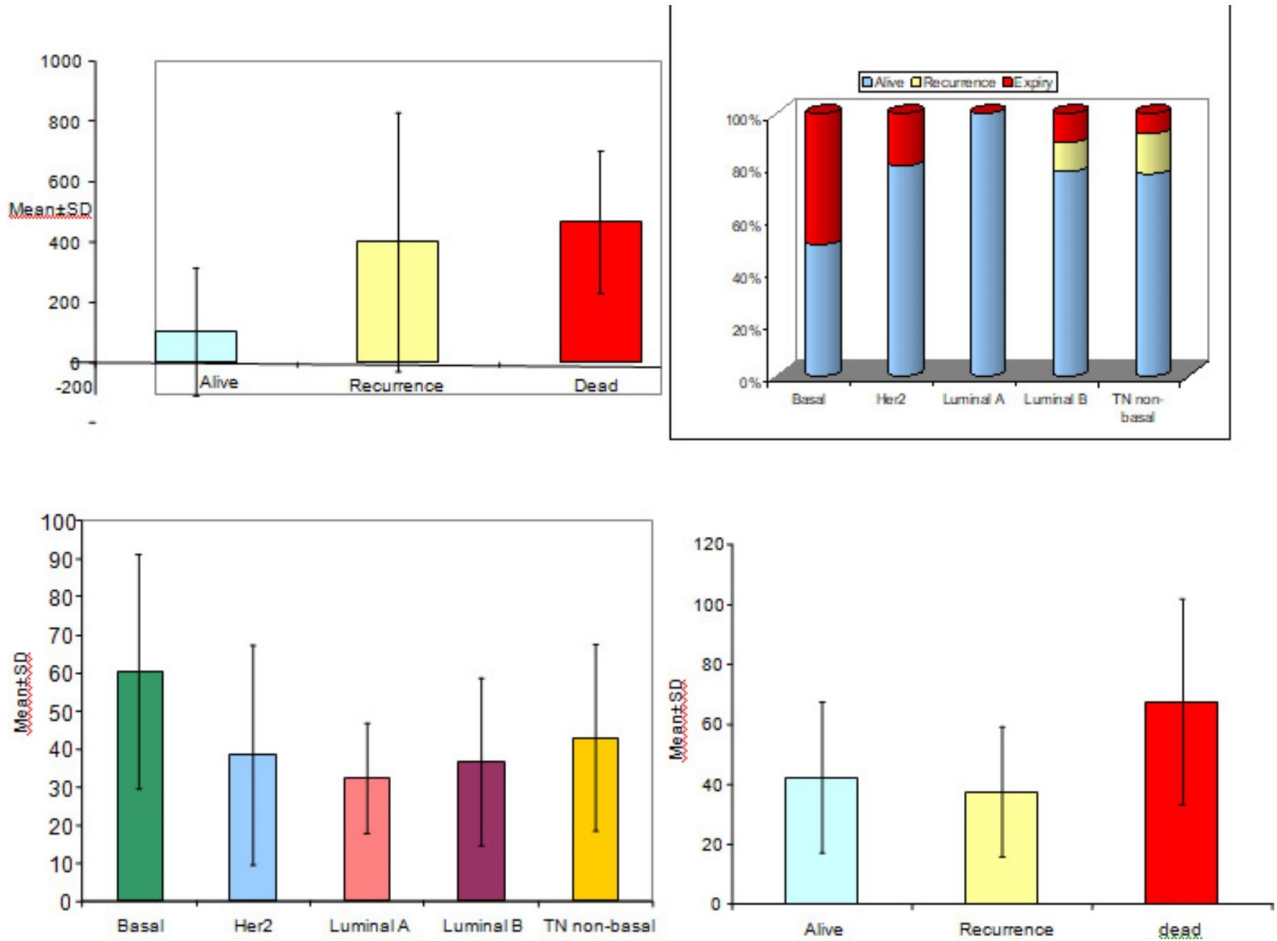


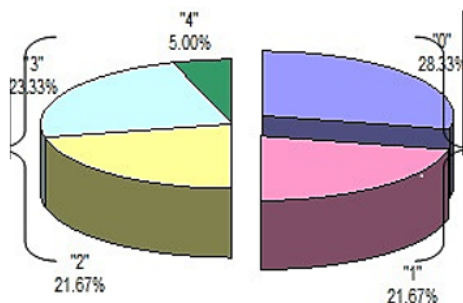
Fig. 1: Comparison of, LU Tumour Size and Outcome (n=32), RU- Molecular Classification with Outcome (n=36), LL- Mean BRCA1 Loss with Molecular Class (n=60), RL BRCA-1(mean Loss) and Outcome LU left upper RU right upper LL left lower RL right lower

Table 1: Expression of BRCA1 (nuclear and cytoplasmic)

Nuclear Expression BRCA1 (N)	
Intensity of expression(a)	Scoring
Absent /faint	0
Moderate / strong	1
Cytoplasmic Expression BRCA1 (C)	
Intensity of cytoplasmic expression (b)	Scoring
No expression	0
Faint	1
Moderate	2
Strong	3
Percentage of tumor nuclei/ cytoplasm stained.(c)	Scoring
< 25%	1
25-50%	2
50-75%	3
>75%	4

BRCA1 EXPRESSION

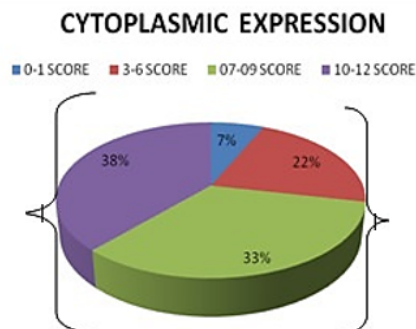
Distribution of Nuclear expression (BRCA1)



2,3,4 were positive for nuclear expression
23.33+21.6+7.5=50%

Loss of nuclear expression 0,1
28.33+21.67=50%

Distribution of cytoplasmic expression (BRCA1)



38% with score (10-12 showed positive cytoplasmic expression

7+22+33=62% of study subpopulation with cytoplasmic loss

Fig. 2: Distribution of expression of BRCA1 (nuclear and cytoplasmic)

Table 2: Distribution on basis of ER PR and HER2 expression(n-114)

Subgroup	ER &/PR+ HER-	ER &/PR+ HER+	ER_PR_HER+	ER-, PR-HER2- Triple negative breast cancer
No. of cases	19 (16.6%)	25 (21.9%)	35 (30.7%)	35 (30.7%)

Table 3: Comparison of Basal like features noted in present study with previous studies

Basal like features	Results expected ^{5,9,10}	Present study
High grade	Grade II and III.	All cases Nottingham's grade II
Basal epithelial gene expressed	CK5/6 & or EGFR +	All cases + for CK5/6 expression ()
Triple negative	Usually triple negative	All cases TNBC
BRCA dysfunction	Present	BRCA1 loss maximum
Early age of diagnosis	Early age of involvement	All cases < 50 yrs
Poor prognosis	Do	Worst prognosis amongst all (50% mortality)

Table 4: Comparison of BRCA1 loss and Nottingham's grade in our study and previous study

Nottingham's grade	Observed value Bal A et al ⁹	Present study	BRCA1 expression loss
Grade I	14.3%	31.85	Minimum
Grade II	57.1%	45.51	Maximum
Grade III	28.6%	37.50	intermediate

mitoses was a major determinant in differentiating grade II and grade III and tubule formation for differentiating grade I and II. In the present study the association between clinical outcome and tumour size was statistically significant. The mean size of tumour in females who succumbed to disease was maximum followed by recurrence group, and least in alive patients. According to a study conducted by Narod S et al. the tumour size was a strong predictor of 15-year survival in both the node-positive and node-negative cancer subgroups.¹⁴ According to the western literature the most

common subtype amongst molecular classification of breast cancer is Luminal A comprising approximately (50%) of total cases.⁵ However in the present study, maximum percentage were of TNBC and Her2 enriched i.e (30.6% respectively) followed by luminal B and luminal A. A study held by Sharma-M et al. in north-eastern Indian population concluded that TNBC accounts for a significant 31.9% of breast cancers in India based on IHC markers.¹⁵ Also Akhtar M et al. in Maharashtra concluded that TNBC forms a large proportion (43.7%) of carcinoma breast patients

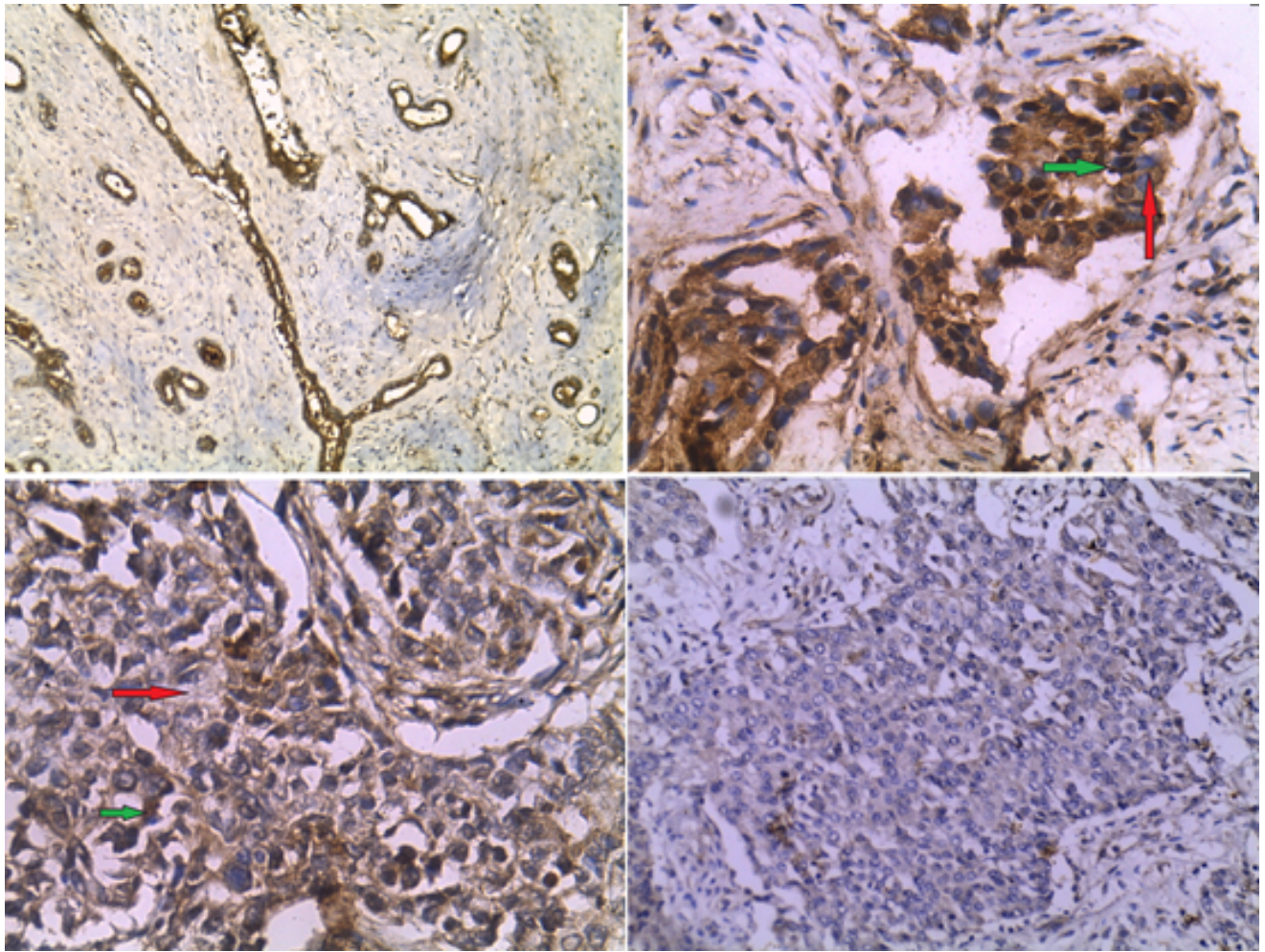


Fig. 3: LU –BRCA1 expression control-strong nuclear and Cytoplasmic positivity of BRCA1 in fibroadenoma. RU-BRCA1 expression analysis case, red arrow –loss of nuclear expression, green arrow-positive nuclear expression- LL- red arrow –loss of Cytoplasmic expression, green arrow-positive Cytoplasmic expression RL-shows severe nuclear and cytoplasmic expression loss of BRCA1(C 4N1)

in a central Indian scenario.¹⁶ This may be contributed to different genotypic compositions of the study population. Further analysis of the TNBC group and its comparison with non TNBC led to certain interesting findings. 77.27% of TNBC belonged to grade II and grade III as compared to 57.5% of non TNBC cases. Also the tumour size in TNBC was found to be larger compared to non TNBC. The results supported past various studies which have stated that TNBC are aggressive set of breast cancer with higher histological grade, larger tumour size, and more often are lymph-node positive. A study by Rebecca Dent et al. in 1601 breast cancer patients found mean tumor size in non TNBC group to be small i.e 2.1 cm against 3 cm in TNBC group with a p value of <0.0001 cm. Also patients in the triple-negative group were more likely to have grade III tumours (66% versus 28%; $P < 0.0001$) and had more frequent tumour metastases.^{17,18}

Prognostically speaking, in our study basal like showed the worst prognosis in which 50% patients succumbed to illness. This was followed by triple negative non basal like category, which showed 15.38% recurrence and 7.7% patients demised. Luminal A showed the best prognosis amongst all with 100% survival. The results are in agreement with the literature which quotes similar findings.¹⁰

BRCA1 mutation has been associated with hereditary breast cancer only. Recent studies indicate that a subgroup of sporadic breast cancer might be associated with the reduction in BRCA1 mRNA levels and protein expression.⁹

Study by Bal A et al. in 2012, found a reduced expression of BRCA1 in 35% and absent/markedly reduced expression in 22.1% of cases of sporadic breast cancer respectively thus concluding role of BRCA1 in sporadic breast cancers.⁹

The sub cellular localisation of BRCA1 protein has been highly debatable. According to few authors its

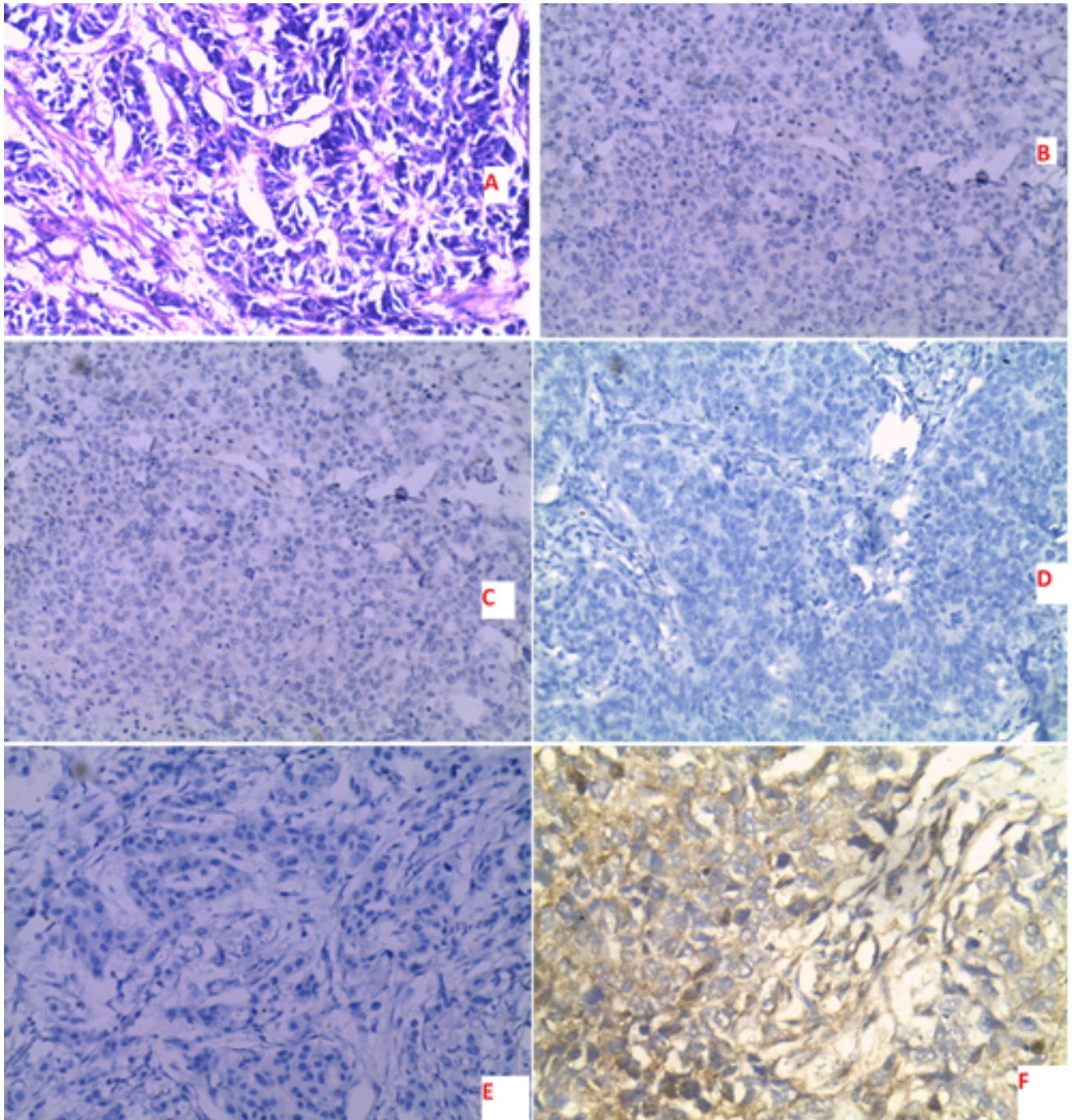


Fig. 4: **A):** TNBC (Basal like) Tumour tissue disposed in tubules H/E stain (200x), **B):** shows ER negativity in tumour cells (200x), **C):** shows PR negativity in tumour cells (200x), **D):** shows HER 2 negativity in tumour cells (200x), **E):** shows ki67L 11% in tumour cells (200x), **F):** shows CK5/6 moderate cytoplasmic positivity in tumour cells (400x)

expression is nuclear while others believe nuclear protein in normal cells but an aberrantly localized cytoplasmic protein in breast and tumour cells. In the present study both cytoplasmic and nuclear expression was seen in both cases and controls, but none of the cases had only nuclear expression. This observation is in agreement with the results of Mulla-F et al. who studied BRCA1 expression in 48 cases of breast cancer and concluded its nuclear + cytoplasmic/only cytoplasmic expression in their study population.¹⁹

In the present study, nuclear expression loss was observed in 50% whereas cytoplasmic expression loss was seen in 61.67%. Before discussing further the demography of BRCA1 loss seen in various subgroups of molecular classification, salient features of basal like needs to be enumerated as this is the subgroup expected to be most associated with BRCA1 expression loss as quoted in western literature¹⁰ and observed in most studies. (Table 3, Figure 4)⁹

Mean BRCA1 expression loss was seen maximum in basal like subgroup (60.42%), followed in decreasing order as: triple negative non basal like (43.01%) %, Her2 neu enriched (38.44%), luminal B (36.61%) and least expression loss was seen in luminal A (32.29)%. The results are in agreement with study of Bal A et al. who have concluded that BRCA1 protein negative tumors are more frequently associated with basal marker positivity and were more often ER receptor negative. Analyzing our data also, we found that maximum BRCA1 protein loss was seen in basal like, followed by triple negative non basal and her 2 enriched all three groups negative for ER positivity. This highlighted that like hereditary cases of breast cancer with BRCA1 mutations, sporadic cases too were more frequently basal phenotype and ER negative. Loss of BRCA1 in sporadic cases suggest that therapeutic targeting of BRCA1 pathway (like PARP inhibitor) might be useful in sporadic cases of breast cancer too. Though BRCA1 loss was statistically non significant yet more loss was seen in grade II and grade III in comparison to grade I (Table 4). Also in our study patients who succumbed to illness showed maximum expression loss as compared to alive/ recurrent group, highlighting the protective role of BRCA1 protein and subsequent consequences upon its mutation.

Hence to conclude Triple negative breast cancers are not only hormone deprived but also treatment deprived subgroups. Recently therapeutic targeting of BRCA1 pathway like PARP inhibitor advocated in hereditary breast cancer, might serve to fill the vacant space in sporadic cases too.

Starting from ER/PR/HER2 with the advent of molecular classification in 2000 and addition of cytokeratin and KI67, the future of molecular classification of breast cancer might lead to addition of BRCA1 expression and open way for novel treatment options and promising outcome.

6. Source of Funding

None.

7. Conflict of Interest

The authors declare that there is no conflict of interest.

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