

Role of Immunophenotypes in Carcinoma Breast

Abstract

Background: Nottingham's modification of Bloom–Richardson histopathological grading system (NGS) for carcinoma breast is a time-tested prognostic indicator; however, of lately, breast cancer has been evaluated through molecular techniques, particularly assessing the gene expression profiling and establishing molecular or immunophenotypes. The present-day utility of NGS needs to be reassessed with the modern predictive markers, this may help refine breast cancer classification specifically to help improve the treatment protocol. **Objective:** The objective was to compare breast cancer immunophenotypes with prognostic factors such as age (based on menstrual status), tumor size, lymph node (LN) status, also to compare the NGS grade with the molecular immunophenotypes of breast cancer. **Materials and Methods:** The present work was carried out in the Histopathology and Immunohistochemistry section of Department of Pathology, of a central Indian medical college and rural hospital from January 2013 to July 2016. It was a prospective analytical study. A total of 114 female patients presenting in the outpatient department of surgery with lump in breast were included in the present study. All patients underwent modified radical mastectomy for tumor resection. Tumor masses and LNs were subjected to routine hematoxylin and eosin staining as well as immunohistochemistry then examined by a senior pathologist. Comparisons were made between molecular immunophenotypes with patient age, tumor size, and LN status, further NGS grade of breast cancer was compared with immunophenotypes. **Results:** The study found that the molecular immunophenotypes when compared with clinical prognostic parameters, i.e; age (based on menstrual status of female), LN involvement in patients of breast carcinoma showed inconsequential correlation, the tumor size showed significant correlation. However, when histopathological grades were compared with molecular immunophenotypes, a significant correlation was seen. **Conclusion:** NGS grade being an excellent predictive prognostic tool should be continued for assessing the grades in breast cancer patients. The molecular markers correlate with the histopathological grading and indirectly aid the oncologist in assessing the aggressiveness, these immunophenotypes are not helpful as suitable prognostic tools. As the molecular phenotypes definitely indicate the hormonal receptor status in breast cancer patients, they become mandatory in guiding oncologists for planning the treatment strategy and protocol.

Keywords: Carcinoma, histopathology, immunohistochemistry, immunophenotypes, lymph node, Nottingham's Modification of Bloom Richardson's Grading system, tumor node metastasis

Introduction

Carcinoma of breast tends to present in an extremely heterogeneous manner as far as the gross and histomorphological features of the breast tumor are concerned. Robust clinical and pathological prognostic and predictive factors to support clinical and patient decision-making for carcinoma breast are available through extensive clinical advances in understanding of the pathology of breast cancer. An estimated 22.9% of invasive cancers in women arise from the breast^[1] and breast cancer comprises of 16% of all cancers occurring in females.^[2] In 2008, breast cancer caused

458,503 deaths worldwide (13.7% of cancer deaths in women and 6.0% of all cancer deaths for men and women together).^[2]

Since the advent of the 19th century, there have been certain prognostic factors which have been well documented for playing a major role in breast cancers occurring in females. These include patient age, axillary lymph node (LN) status, tumor size, histological features (especially histological grade and lymphovascular invasion), hormone receptor status. Presently, the tumor tissue from breast cancer has been evaluated through molecular techniques, particularly assessing the gene expression profiling, and establishing molecular or immunophenotypes, this has helped refine

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breast cancer classification specifically to help improve the treatment protocol. Although the precise role of these newer techniques in the daily management of patients with breast cancer continues to evolve, it is clear that they have the potential to provide value above and beyond that provided by the traditional clinical and pathological prognostic and predictive factors.

The molecular gene profiling has come out with many new immunophenotypes in tumor tissue of breast cancer and these have well aided in the assessment of histopathological grading of breast cancer. Blending the histopathologic grades with the immunophenotypes/molecular subtypes in breast cancer tissue, has been assessed as one of the best combinations for prognostication and deciding treatment protocol in these patients. The combined approach forms the basis of a number of schema used to group patients into various risk categories such as the St. Gallen criteria,^[3] the National Institute of Health consensus criteria,^[4] and the Nottingham Prognostic Index.^[5]

The present study was undertaken to analyze and assess the relationship between Nottingham's modification of Bloom–Richardson histopathological grading system (NGS) and certain timebound prognostic factors such as age, tumor size as well as nodal status and further correlate the NGS as well as the mentioned prognostic factors with the newly established immunophenotypes in breast cancer based on the St. Gallen's consensus. This study finds an important need to establish whether the conventional NGS through histopathology still finds itself sufficient and significant within the conventional histopathological reporting protocol, in a world of molecular advancements or is it time to reconsider its gold standard.

Keeping these points in mind, the present study was designed with an aim to establish a correlation between NGS grading and the immunophenotype (molecular subtype) biomarkers of breast carcinomas as well as other prognostic factors such as age (based on menstrual status), tumor size, and LN status in breast cancers.

Materials and Methods

The present work was carried out in the Histopathology and Immunohistochemistry section of Department of Pathology, central Indian medical college and rural hospital from January 2013 to July 2016. It was a prospective analytical study. A total of 114 female patients presenting in the outpatient department of surgery with lump in breast were included in the present study.

These patients were examined and suspected patients for carcinoma breast under went fine-needle aspiration cytology (FNAC) or core needle biopsy. The patients diagnosed with carcinoma breast on FNAC or core needle biopsy were admitted as inpatients of Department of Surgery. All patients underwent modified radical mastectomy for tumor resection. All these resected

specimens were grossed as per standard protocol. Multiple sectioning from tumor mass was done. A minimum of ten LNs were resected from the specimen. Formalin-fixed, paraffin-embedded blocks of tumor masses, LNs were subjected to routine hematoxylin and eosin (H and E) staining and immunohistochemical profiling for molecular immunophenotypes.

The histopathological examination of the sections from this tumor mass was done and the tumor was divided into three grades as per the scoring system based on histopathological parameters such as tubule formation, nuclear pleomorphism, and mitosis. This applied system is known as the NGS. The grades were divided as per score as Grade I (3–5) [Figure 1a], Grade II (6–7) [Figure 1b], and Grade III (8–9) [Figure 1c].

A minimum of ten axillary LNs were resected from each specimen. The resected LNs were looked for the evidence of LN metastasis [Figure 1d] on histopathologic examination.

Standardized immunohistochemical staining procedure was followed using the Dako estrogen receptor (ER), progesterone receptor (PR), HER 2 NEU, and MIB-1 monoclonal antibody (for ki-67 antigen) kit for performing the staining process. The molecular subtyping of the immunohistochemical stained slides was done and these sections were categorized into four molecular subtypes namely Luminal A, luminal B, HER 2 NEU positive,

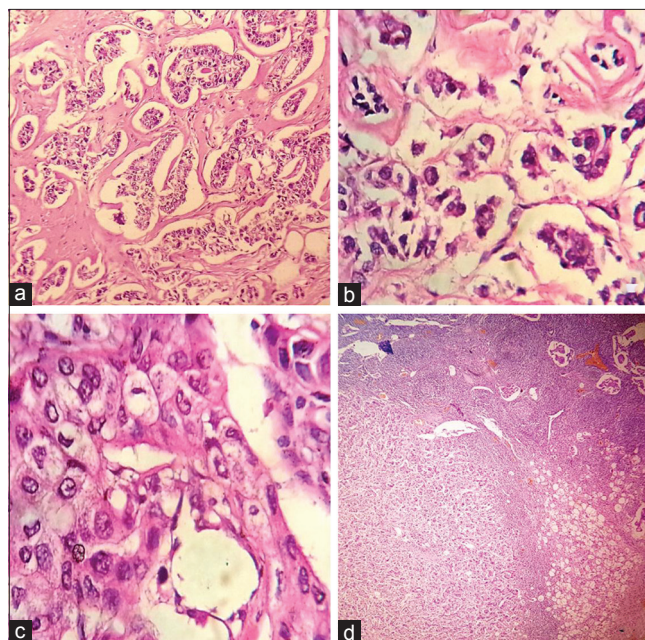


Figure 1: Hematoxylin and eosin-stained section ($\times 40$ view) from tumor mass of breast shows histopathological features of infiltrating ductal carcinoma (not otherwise specified type), Nottingham's modification of Bloom–Richardson histopathological grading system Grade I (a), Nottingham's modification of Bloom–Richardson histopathological grading system Grade II (b), Nottingham's modification of Bloom–Richardson histopathological grading system Grade III (c). H and E, section $\times 4$ view of lymph node shows metastatic deposits of ductal malignancy (d)

and triple-negative breast carcinoma (TNBC) based on the positivity for ERs [Figure 2a], PRs [Figure 2b], Ki 67 positive [Figure 2c], and Her 2/Neu transmembrane staining [Figure 2d].

Positive/negative controls were selected for ER, PR, and HER 2/NEU, Ki67. Known breast tissue, positive for ER, PR, and Her2 was taken as the positive control, similarly known TNBC tissue was taken as negative control. Positive control for Ki-67 comprised of proliferating follicles in a reactive LN and Ki67 negative control was known tissue from lipoma.

To quantify the hormonal status of the tumor mass (ER and PR), the Allred score was used. The Allred score is the sum of the proportion score (proportion of stained nuclei of cells) and the intensity score (intensity of the stained nuclei). Positive interpretation requires at least 1% of tumor cells showing positive nuclear staining of any intensity. Receptor negative is reported if <1% of tumor cells show staining of any intensity. For HER 2/NEU, transmembranous staining was studied in the tumor cells and further scoring was done based on the percentage of tumor cells stained and whether the staining was complete membrane staining or incomplete. Positive for HER2/NEU was consider with strong intramembranous staining along with >30% cells positive. Similar methodology was performed for Ki-67 antigen, where percentage of nuclear positivity in 100 tumor cells was calculated and further categorized as positive (when it was $\geq 14\%$) and negative when it was <14%.

H and E-stained slides as well as immunohistochemical slides were then examined by a senior pathologist.

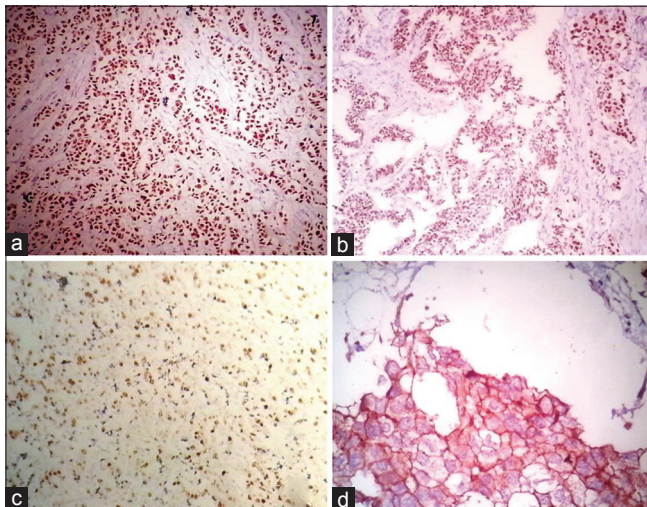


Figure 2: Immunohistochemistry-stained section ($\times 10$ view) from breast tissue mass shows intense brown color-stained nuclei signifying estrogen receptor positive (a) and progesterone receptor positive; (b) immunohistochemistry-stained section ($\times 10$ view) from breast tissue mass shows intense brown nuclear positivity signifying Ki-67 labeling index >14%; (c) immunohistochemistry-stained section ($\times 40$ view) from breast tissue mass shows strong complete membrane staining in >30% signifying HER2/Neu positive (d)

Inclusion criteria

All cases reported to the surgical outpatient department with breast lump and subsequently diagnosed as breast carcinoma on FNAC/core needle biopsies and operated by modified radical mastectomy and subsequently reported as invasive ductal carcinoma (IDC), not otherwise specified on histopathological examination were considered as cases for the present study.

Exclusion criteria

- Patients with previous lumpectomy
- Patients who underwent neoadjuvant chemotherapy
- Patients with recurrence
- Patients with coexisting malignancy
- Patients on chemotherapy/radiotherapy
- Patients not compliant for the study
- Patients with noncarcinomatous breast malignancy
- IDC (special type)
- Invasive lobular carcinoma
- All patients >55 years of age presenting with menstrual irregularities.

Approach and methodology in the present study

At first, 114 patients were divided based on their menstrual status into three age groups which were premenopausal (<48 years), menopausal (48–55 years), and postmenopausal (>55 years). The second parameter was size of the tumor mass. On gross examination the tumor mass was categorized into three groups based on the dimensions of tumor mass in greatest dimension which were Group I (<2 cm), Group II (2–5 cm), and Group III (>5 cm), tumor sizes were categorized according to the tumor node metastasis staging protocol. The histopathological examination of the sections from this tumor mass was done based on the NGS. The axillary LNs were distributed into two categories based on the positivity for infiltration by malignant epithelial cells and reactive lymphadenitis.

Patients were categorized on immunohistochemistry according to four molecular subtypes, i.e., Luminal A, Luminal B, Her2/Neu, and TNBC and then correlated with significant prognostic indices as follows: age distribution [Table 1], tumor size [Table 2], and LN status [Table 3]. Finally, the NGS itself was compared and correlated to the molecular subtypes [Table 4].

Statistical analysis for this study was carried out using multinomial regression analysis as well as Pearson's Chi-square test. Correlation was carried out and "*P*" <0.05 was considered significant to conclude the final statistical interpretation.

Observation and Results

Multinomial regression analysis was carried out to compare immunophenotypes versus prognostic markers (age, tumor size, and LN status) observation analysis is reflected in table with log odds [Figure 3].

**MULTINOMINAL LOGISTIC REGRESSION:
(WITH AGE AND TUMOR SIZE AS CONTINUOUS VARIABLES)**

```
. mlogit ihctype age tumorsize lymphnodestatus_cat, base(2)
```

Iteration 0: log likelihood = -155.0642
Iteration 1: log likelihood = -148.05993
Iteration 2: log likelihood = -147.97887
Iteration 3: log likelihood = -147.97883
Iteration 4: log likelihood = -147.97883

Multinomial logistic regression Number of obs = 114
LR chi2(9) = 14.17
Prob > chi2 = 0.1164
Log likelihood = -147.97883 Pseudo R2 = 0.0457

ihctype	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]
Her2_Positive					
age	.0180074	.0225581	0.80	0.423	-.0261256 .0623005
tumorsize	.3359145	.1346556	2.49	0.013	.0719945 .5998346
lymphnodestatus_cat	.236263	.5903938	0.40	0.689	-.9208877 1.393414
_cons	-2.754423	1.283145	-2.15	0.032	-5.26934 -.2395057
Luminal_A (base outcome)					
Luminal_B					
age	.0095217	.0213497	0.45	0.656	-.0323229 .0513663
tumorsize	.0616741	.1374575	0.45	0.654	-.2077376 .3310857
lymphnodestatus_cat	.6053048	.5560266	1.09	0.276	-.4844874 1.6950897
_cons	-.8967948	1.175983	-0.76	0.446	-3.201679 1.408089
TNBC					
age	.0033734	.0257465	0.13	0.896	-.0470088 .0538356
tumorsize	.2954057	.1468482	2.01	0.044	.0075085 .5032228
lymphnodestatus_cat	.24381	.6668439	0.37	0.715	-1.06318 1.5508
_cons	-2.291525	1.42964	-1.60	0.109	-5.093567 .5105174

Figure 3: Immunophenotypes versus prognostic markers (age, tumor size, and lymph node status)

The multinomial regression analysis showed the following findings: A 1-year increase in the age is associated with a 0.009 increase in the relative log odds of being in Luminal B versus Luminal A. A 1-year increase in the age is associated with a 0.018 increase in the relative log odds of being in HER2 versus Luminal A. A 1-year increase in the age is associated with a 0.003 increase in the relative log odds of being in TNBC versus Luminal A. A 1-cm increase in the tumor size is associated with a 0.061 increase in the relative log odds of being in Luminal B versus Luminal A. A 1-cm increase in the tumor size is associated with a 0.335 increase in the relative log odds of being in HER2 versus Luminal A. A 1-cm increase in the tumor size is associated with a 0.295 increase in the relative log odds of being in TNBC versus Luminal A.

The relative log odds of being in Luminal B versus Luminal A will increase by 0.60 if moving from the lowest level of LN status (LNS==negative) to the highest level of LN status (LNS==positive). The relative log odds of being in HER2 versus Luminal A will increase by 0.23 if moving from the lowest level of LN status (LNS==negative) to the highest level of LN status (LNS==positive). The relative log odds of being in in TNBC versus Luminal A will increase by 0.24 if moving from the lowest level of LN status (LNS==negative) to the highest level of LN status (LNS==positive).

Interpretation

1. Under the age category: The observations suggests that the age category did not correlate well with the immunophenotypes, when age groups were analyzed as a continuous variable in the multinomial regression analysis
2. Under the tumor size category: The observations suggests that the tumor size correlated significantly with the immunophenotypes, when tumor size were analyzed as a continuous variable in the multinomial regression analysis
3. Under the LN status category: The observations suggests that the LN status category did not correlate well with the immunophenotypes, in the multinomial regression analysis.

Discussion

Since the turn of the 19th century, breast cancer has been under research and there has been extensive findings emanating from these researches which have established certain important markers to predict the prognosis among breast cancer patients. For many years, the cancer of breast has been scaled with respect to prognostic factors such as the reproductive age of the patient, the tumor size as well as the LN metastatic status. Patients presenting with cancer of breast initially were subjected to more or less similar surgical as well as chemotherapy protocol. With advancements in our understanding at the molecular levels, the hormonal receptor status as well as the newer entry of molecular gene profiling the outlook toward prognostication and management of these cancers has brought in a revolution in the medical field. Presently utilized newer molecular classification incorporates the utility of ER, PR, Ki-67, Her 2 neu, and their categorization based on their positivity into Luminal A, Luminal B, Her 2 positive, and TNBCs. This regrouping of breast cancers basically aims to improve the overall treatment as well as the management protocol of breast cancers.

The major findings at the genetic levels (epigenetic and transcriptome included) have compelled the histopathologist to revisit the conventional classifications of breast cancers. Even the NGS and its prognostic importance has come into interrogation. Hence considering the new genetic categorization as well the hormonal makeup of the breast tumor mass, the treatment protocols have been reestablished.

The advancement of molecular techniques such as gene expression profiling, has created a kind of uncertainty among the pathologists as far as the histopathological typing, grading and classification of breast carcinomas are concerned based on the conventional serotypical surgicopathological techniques and protocols. The present-day management and prognostication are being realigned based on these molecular gene profiles as well as the hormonal receptor status. We still need to know

Table 1: Correlation between molecular subtypes and age (based on menstrual status) in carcinoma breast patients

Molecular subtypes versus Age group	Luminal A (%)	Luminal B (%)	HER 2/NEU (%)	Triple negative (%)	Total	χ^2
Premenopausal	18 (56.25)	17 (50)	13 (43.33)	10 (55.55)	58	3.00
Menopausal	7 (21.87)	5 (14.70)	6 (20)	4 (22.22)	22	$P=0.80$ (NS)
Postmenopausal	7 (21.87)	12 (35.29)	11 (36.66)	4 (22.22)	34	
Total	32 (28.07)	34 (29.82)	30 (26.31)	18 (15.78)	114	

NS: Not significant

Table 2: Correlation between molecular subtypes and tumor size in carcinoma breast patients

Molecular subtypes versus Tumor size	Luminal A (%)	Luminal B (%)	HER 2/NEU (%)	Triple negative (%)	Total	χ^2
<2 cm	2 (6.25)	1 (2.9)	0	0	3	17.17
2-5 cm	24 (75)	25 (73.52)	12 (40)	9 (50)	70	$P=0.0087$ (S)
>5 cm	6 (18.75)	8 (23.52)	18 (60)	9 (50)	41	
Total	32 (28.07)	34 (29.82)	30 (26.31)	18 (15.78)	114	

S: Significant

Table 3: Correlation between molecular subtypes and lymph node status in carcinoma breast patients

Molecular subtypes versus Lymphnode status	Luminal A (%)	Luminal B (%)	HER 2/NEU (%)	Triple negative (%)	Total	χ^2
Negative	23 (71.87)	19 (55.88)	16 (53.33)	10 (55.55)	68	2.81
Positive	9 (28.12)	15 (42.85)	14 (46.66)	8 (44.44)	46	$P=0.42$ (NS)
Total	32 (28.07)	34 (29.82)	30 (26.31)	18 (15.78)	114	

NS: Not significant

Table 4: Correlation between Nottingham grading system (histopathological grading) and molecular subtypes in carcinoma breast patients

Molecular subtypes versus histopathological Grade	Luminal A (%)	Luminal B (%)	HER 2/NEU (%)	Triple negative (%)	Total	χ^2
Grade I	16 (61.53)	7 (26.92)	3 (11.53)	0	26	54.23
Grade II	12 (29.26)	21 (51.21)	5 (12.19)	3 (7.31)	41	$P=0.0001$ (S)
Grade III	4 (8.51)	6 (12.76)	22 (46.80)	15 (31.91)	47	
Total	32 (28.07)	34 (29.82)	30 (26.31)	18 (15.78)	114	

S: Significant

through research and analysis the amount of weightage and significance we need to give to these recently established molecular classifications of breast cancers.

Present study in comparison with other studies

Molecular (immunophenotypes) subtypes in breast cancer in comparison to patient age, tumor size, lymph node status and Nottingham's modification of Bloom–Richardson histopathological grading system (histopathological grades)

In our study, a total of 114 female patients were diagnosed with carcinoma breast. These molecular subtypes were compared with the age (as per the menstrual status of the women diagnosed with breast cancer). The study had, following distribution of premenopausal patients, 56.25% of Luminal A subtype and 50% of Luminal B subtype, contrastingly there were less percentage of postmenopausal

patients in luminal positive subtypes. However, among the high-grade breast malignancy categories of Her 2 neu and Triple negative, the distribution of premenopausal age group patients was high, i.e., 13 out of 30, (43.33%) and 10 out of 18 (55.55%) respectively in both categories. Thus suggesting the fact that aggressive cancers occur in younger age group.

Our study had similar findings to the studies of Su *et al.*, Inwald *et al.*, Najafi *et al.*, Alnegheimish *et al.*,^[6-9] where significant difference was found in breast cancer molecular subtypes in regard to age, Luminal A subtype of breast cancer was commonly found in >50-year-old women. TNBC/basal-like cancer was mostly diagnosed among <50-year-old women.

In our study of 114 female patients diagnosed with carcinoma breast, comparisons were made between the

molecular subtypes and tumor size. The present study had the following distribution of patients in all the four molecular subtypes as per size of tumor mass. We observed that luminal A and luminal B molecular subtypes both of which are hormone receptor subtypes having (ER+, PR+) were seen to occur in small size of tumorous growth of breast cancers. Conversely, the study showed molecular subtypes such as Her 2 neu and triple negative were positive in large size tumors, suggesting the fact that hormone receptor status in tumoral tissue size has significant relationship. The study findings suggested the fact that with the proliferating tumor cells and larger size of tumor mass the hormone receptor status was found to be negative or was found positive for estimated glomerular filtration rate and in tumors of low-grade aggressiveness such as Luminal A and Luminal B type the tumor tissue was found to be positive for ER/PR receptors and hence had increased receptivity to these hormones.

Our study found statistical significance between tumor size and all molecular subtypes, similar to the findings of Su *et al.*, and Inwald *et al.*, and Alnegheimish *et al.*

In our study, comparison was done of molecular subtypes with the number of patients positive for metastasis versus patients who had no nodal metastasis (reactive lymphadenitis). The LN status in the given mastectomy specimen with breast mass was considered as a significant prognostic factor.

In our study, the luminal A and luminal B categories (low-grade subtypes), patients were found to have more reactive lymphadenitis and low predominance of metastatic deposits. These findings were similar to the study findings of Widodo *et al.*^[10] Su *et al.*, Inwald *et al.*, However, the study observed that when the molecular subtypes (all four categories) were compared with the LN status (metastasis or reactive lymphadenitis) in the patients, there was no strong correlation between LN metastasis with a high-grade molecular subtype (her 2 neu and triple negative). These findings were similar to the findings of Bennis *et al.*,^[11] Najafi *et al.*, Alnegheimish *et al.*

In our study, the cases were divided into three grades depending on the histopathological scoring of breasts carcinoma, the NGS (histopathological grading) of tumor mass has been considered in the present study as a most important parameter for diagnosis and prognostication of breast cancer.

When the NGS was compared to molecular subtypes, the following findings were made; it was seen that majority of histopathological Grade I tumors matched with the molecular subtype of luminal A, i.e. 61.53% of cases of luminal A subtypes fell into Bloom–Richardson (BR) Grade 1 category, Similarly, the majority of histopathological Grade II tumors matched with the molecular subtype of luminal B, i.e. 51.21% of cases of luminal B subtype fell

into the BR Grade 2 category, suggesting the fact that the cell proliferation index and tumor aggressiveness of luminal A and B category is of low potential akin to the low histopathological grade (BR Grade 1 and Grade 2) tumors. The majority of histopathological Grade III tumors matched with the molecular subtypes of HER 2 Neu and TNBCs, a total of 37 cases out of 47 (78.71%), i.e., 46.80% and 31.91% cases, respectively, were found in BR Grade 3 category, suggesting the fact that the cell proliferation index and tumor aggressiveness of HER 2 NEU and triple negative category is of extremely high potential akin to the high histopathological grade (Grade III) tumors.

These findings were similar to the study findings in the following studies: Widodo *et al.*, Su *et al.*, Inwald *et al.*, Alnegheimish *et al.*, Najafi *et al.*, and Bennis *et al.*

Conclusion

The study found that the basic immunophenotypes markers of breast carcinoma have an excellent correlation with the histopathologic grades; NGS can be considered as a reliable alternative tool, for assessment of the endocrine status in cases of breast cancer in rural setups.

The NGS is a validated alternative to molecular tests with special utilities in rural parts of the globe and India in particular, where access to new molecular technology is not currently available or likely to become available in the near future.

The molecular (immunophenotypes) subtypes of breast cancer are excellent molecular level tissue markers and should be utilized for therapeutic decisions on an individual case-to-case basis.

Molecular assays and NGS should complement rather than compete with each other. We conclude that the assessment of histological grade NGS is an important determinant of breast cancer prognostication and should be incorporated in algorithms with immunophenotypes to define therapy for patients with breast cancer.

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Conflicts of interest

There are no conflicts of interest.

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