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Original Research Paper

Pathology

COMPARISON OF CLINICOPATHOLOGICAL PARAMETERS WITH HISTOLOGICAL AND MOLECULAR CLASSIFICATION OF BREAST CANCERS

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ABSTRACT

BACKGROUND: This is a retrospective study of 60 cases, to detect the expression of ER, PR, HER2neu, CK5/6 and Ki67 proliferation index in breast carcinomas by immunohistochemical method and to determine the newer molecular classification. Few patients have recurrence inspite of being diagnosed under the category of low risk and few do well in the high risk group which can be attributed to the molecular level differentiation.

AIM: The aim of this study is to categorize the patients under molecular classification, and to compare the clinicopathological parameters with it and to denote the significance of targeted therapy.

MATERIALS AND METHODS: A retrospective study of detecting the expression of the above said markers in modified radical mastectomy specimens received at a tertiary care centre during the period from January 2015 to June 2018. A total of 60 cases which included 30 of IDC NST and 30 cases of special variants were selected for immunohistochemical analysis.

RESULTS: Out of the 60 cases studied, the most common was found to be the luminal A type comprising 37% and the least common was the luminal B and hybrid types each comprising 8%. The most common grade for HER2 was Grade III (50%). The association of histological grade with the molecular classification was statistically significant with the p value of 0.01. Basal type (56%) had the highest incidence of N3 stage. ER, PR, HER2 neu, CK5/6 expression and proliferation index with Ki67 had a statistically significant association with the molecular classification. High proliferation index (>14%) with Ki67 was noted in Luminal B, Basal and Hybrid types. 78% of the total 60 cases were alive and healthy. One death was reported in HER2, Hybrid and Basal types. The negative kappa value obtained while studying the agreement between the histopathological and molecular classification, indicates that the agreement is worse than chance and hence the importance of molecular classification is substantiated for the targeted therapy.

KEYWORDS : Molecular Classification, Histopathological classification, Immunohistochemistry, Targeted Therapy.

INTRODUCTION

Of all the female cancers, breast carcinoma comprises 16% of the total cases and is one of the most commonly diagnosed cancers worldwide (Sarkar and Mandal, 2011). Although it is the most common cause for cancer related deaths in developing countries overtaking the cervical cancers with relatively poor survival. Its incidence in India is 30-33% per 1,00,000 women and the relative risk is 0.033(1 in 30) (Chang et al., 2010). Early diagnosis and treatment will certainly reduce the mortality rates. Breast cancers are broadly classified into ductal carcinomas and lobular carcinomas. The molecular markers in breast cancer have gained importance not only as prognostic indicators but also as predictors to therapeutic response. Especially the steroid receptors -estrogen receptor (ER), progesterone receptor (PR), HER2 neu, CK5/6 and Ki67 have gained increasing interest (Andre and Pusztai, 2006)

Recent advances in breast pathology that examines the RNA, DNA and proteins of malignant cells have provided an algorithm for the new molecular classification of breast cancers (Andre and Pusztai, 2006; Peppercorn J, et al., 2008). Based on the gene expression profiling, five major patterns of gene expression has been identified: Luminal A, Luminal B, HER2 type, Basal and unclassified types (Andre and Pusztai, 2006; Peppercorn J, et al., 2008).

AIMS AND OBJECTIVES

In this study of 60 cases which included invasive ductal carcinoma no special type (IDC NOS) and its special variants, an attempt has been made to evaluate the hormonal status and proliferation index by immunohistochemistry.

The aim of the study is to identify the relative frequency and distribution of breast carcinoma in the south Indian population. and to study the histomorphological features of breast carcinoma including grade, lymph node status, lymphovascular invasion and necrosis and correlate with the expression of ER, PR, HER 2 neu, CK5/6 and Ki67 in invasive breast carcinomas and thereby subtyping the breast cancers as Luminal type A, Luminal type B, HER2 neu type and Basal type. This subtyping helps to denote the significance of molecular classification in the treatment of the patients.

MATERIALS AND METHODS

Sixty patients with invasive breast cancers cases diagnosed and treated at a tertiary care hospital during the period between Jan 2015 to Jun 2018 were included. Clinical details of all mastectomy cases were collected from the records and 60 cases were selected based on the histological diagnosis that included invasive breast carcinomas no special type (ductal and lobular) and special types as medullary, mucinous, papillary, apocrine and metaplastic carcinomas irrespective of the age and sex excluding the benign lesions and mesenchymal tumors. Formalin fixed paraffin embedded tissues were processed by standard technique and the slides were stained with hematoxylin and eosin. All the histopathological slides were retrieved, re-evaluated and graded using the Nottingham modification of the Scarff Bloom Richardson Grading system.

Ten cases of each grade from Invasive ductal carcinoma NST [Fig1, Fig 2, Fig 3] and 5 cases from special type as medullary [Fig 4], metaplastic [Fig 5], mucinous [Fig 6], apocrine [Fig 7], papillary [Fig 8] and invasive lobular [Fig 9] were randomly selected from the total cases and their representative tissue samples were subjected to immunohistochemical analysis of 5 markers which includes ER [Fig 10], PR [Fig 11], H2N [Fig 12], CK5/6 [Fig 13] and Ki67 [Fig 14] with appropriate positive and negative controls. Slides were evaluated and scoring was given. The results were recorded with photographs.

IMMUNOHISTOCHEMICAL EVALUATION

Hormone receptors like estrogen and progesterone receptor, when expressed show a nuclear positivity. The number of cells expressing and their intensity of staining is scored as two values and a composite score based on percentage plus intensity of more than 2 is considered to be positive. HER2neu expression is demonstrated in tumor cells as cytoplasmic membrane positivity and its intensity and number of tumor cells expressing is graded as 1+, 2+ and 3+.

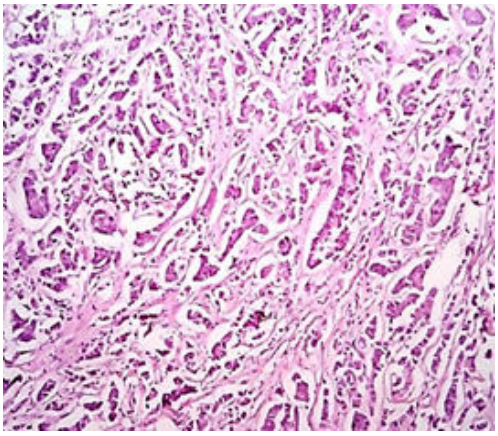

Figure 1: Invasive ductal carcinoma NST Grade I; H&E, 40x

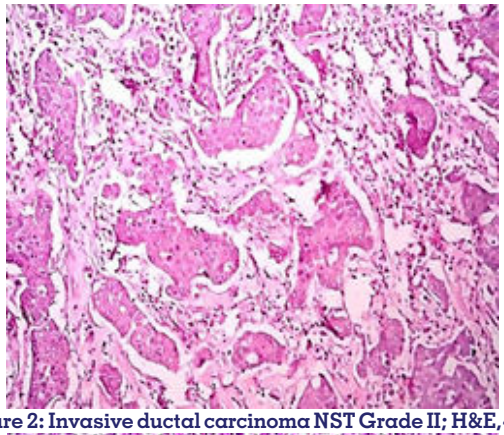

Figure 2: Invasive ductal carcinoma NST Grade II; H&E, 40x

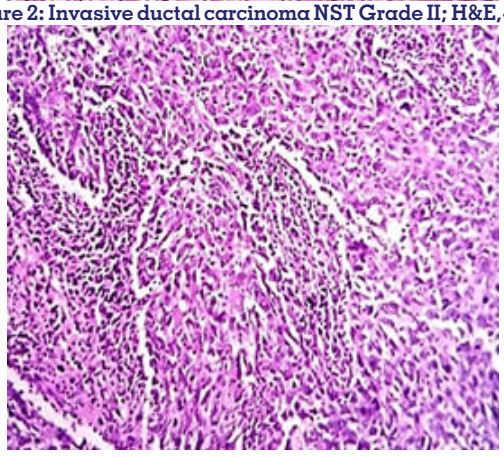

Figure 3: Invasive ductal carcinoma NST GradeIII; H&E, 40x

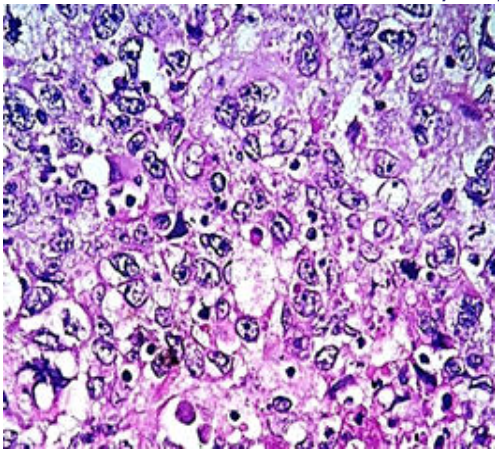

Figure 4: MEDULLARY CARCINOMA Tumor cells in syncytial pattern with marked nuclear pleomorphism. H&E, 400x

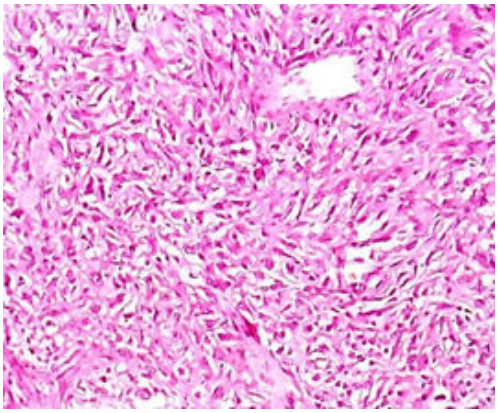

Figure 5: METAPLASTIC CARCINOMA:Nests of tumor cells along with spindled cells. H&E, 400x

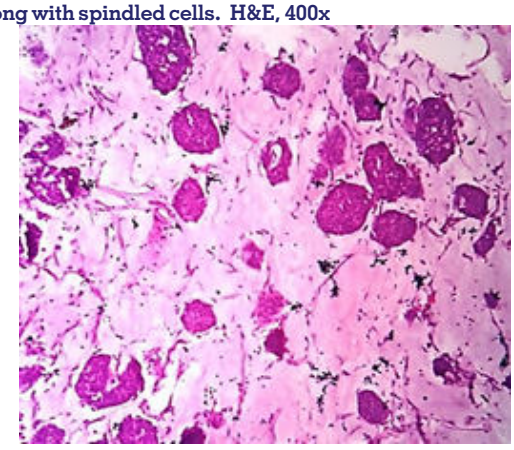

Figure 6: MUCINOUS CARCINOMA: Islands of tumor cells floating in mucin pools. H&E, 400x

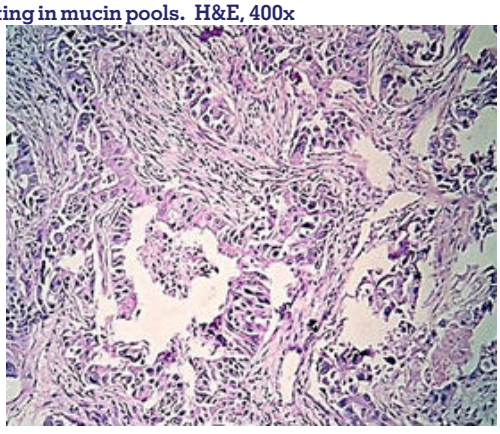

Figure7:APOCRINE CARCINOMA: Nests of tumor cells with abundant eosinophilic cytoplasm.H&E, 40x

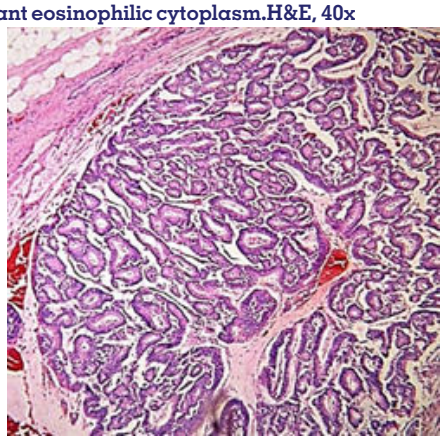

Figure 8:PAPILLARY CARCINOMA: papillary fragments with central fibrovascular core. H&E, 40x

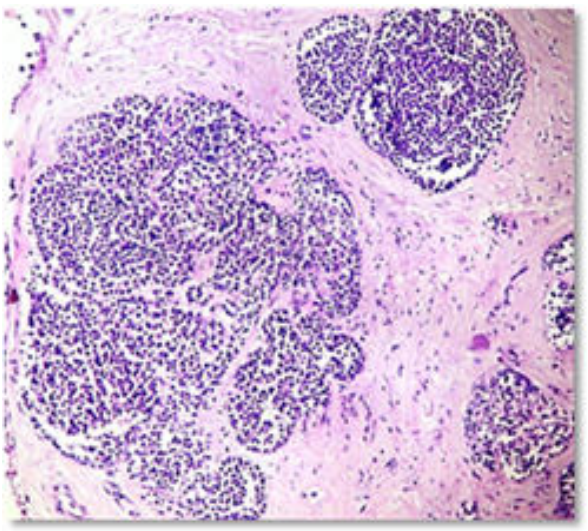

Figure 9: LOBULAR CARCINOMA-Neoplastic cells seen in lobules. H&E, 40x

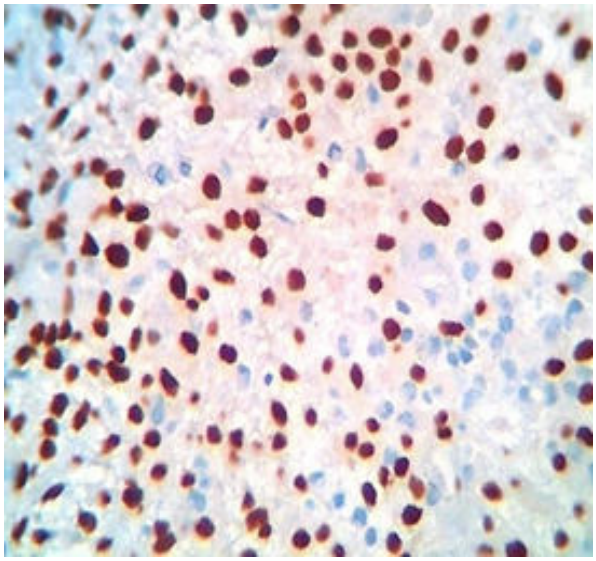

Figure 10: Positive nuclear staining (5+3) for estrogen receptor. H&E, 100x

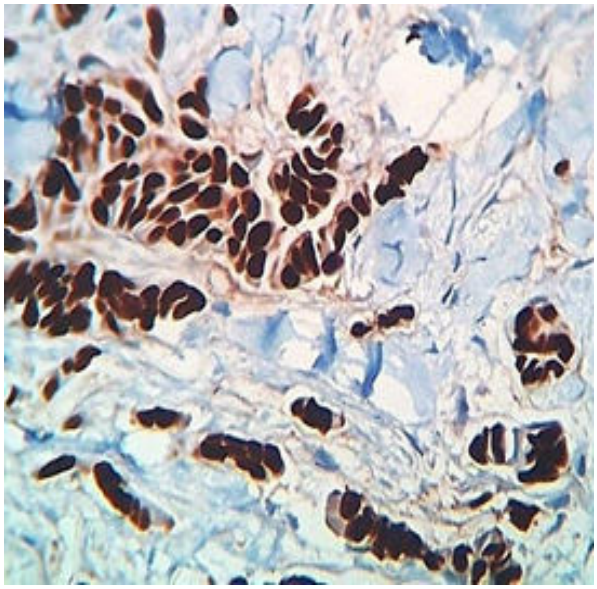

Figure 11: Positive nuclear staining (5+3) for progesterone receptor. H&E, 400x

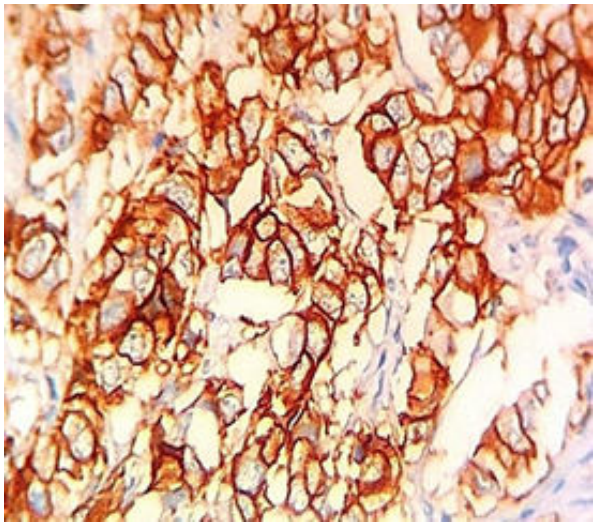

Figure 12: Positive cytoplasmix membrane staining (3+) for Her2 Neu receptor. H&E, 400x

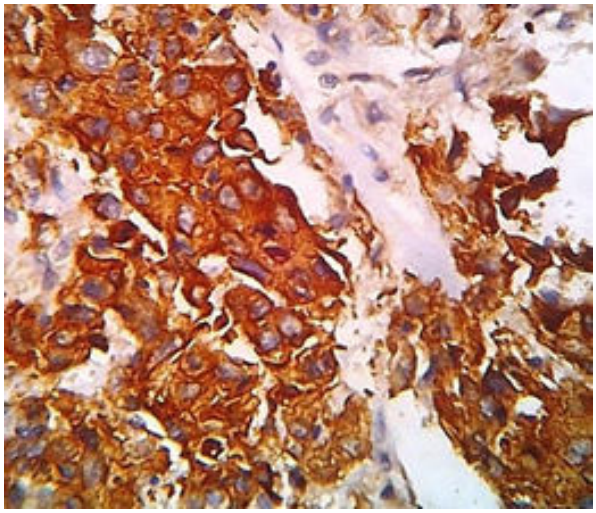

Figure 13: Positive cytoplasmic staining for CK 5/6. H&E, 400x

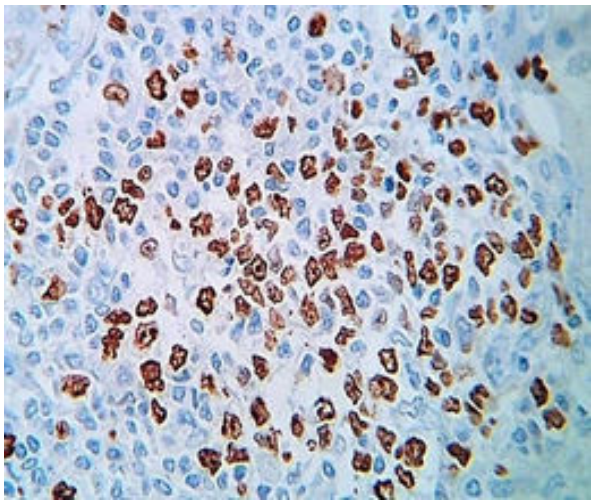

Figure 14: Positive nuclear staining for Ki 67 proliferastion index. H&E, 400x

CK 5/6 scoring was done based on the criteria proposed by Smedts et al and Ordonez et al in their studies on gynaecological malignancies. The scoring was based on the percentage of positive cells. For Ki67 nuclear reactivity is taken into account, which is recorded as continuous variables,

based on the proportion of positive tumor cells (0%- 100%) irrespective of the staining intensity. They are regarded as high when >14% and low when <14% (Table 1, 2 & 3).

Table 1: Er/pr Scoring

	INTENSITY OF STAINING	PROPORTION OF CELLS STAINED	INTERPRETATION
0	NONE	NONE	NEGATIVE
1	WEAK	<1/100 CELLS	NEGATIVE
2	MODERATE	1-10/100 CELLS	POSITIVE
3	STRONG	11-33/100 CELLS	POSITIVE
4		34-68/100 CELLS	POSITIVE
5		>68/100 CELLS	POSITIVE

Total Score: 0-8; Out Of Any Score <2 Is Negative And Have A Negligible Chance Of Response.

Table:2 Staining Pattern And Her2 Neu Scoring

Staining pattern	Score	Her 2/ neu Assesment
No staining or membrane staining Observed in <10% of tumor cells	0	Negative
A faint/barely perceptible membrane staining observed in >10% of the tumor cells	1+	Negative
A weak to moderate complete membrane staining observed in >10% of tumor cells	2+	Positive
A strong complete membrane staining observed in >30% of the tumor cells	3+	Positive

Table 3: Ck5/6 Scoring

Score1	25% of the tumor tissue show positivity
Score 2:	26-50% of tumor cells show positivity
Score3:	51 -75% of the tumor tissue show positivity.
Score 4:	76-100% of tumor cells show positivity

STATISTICAL ANALYSIS:

Molecular subtypes were derived and compared with the clinico pathological parameters with the SPSS version 17 software. The Mcnemar's test is done by a 2x2 classification table. This is done to test the difference between paired proportions. Here in this study it is done on the same set of patients who serve as their own control, based on the "before and after" design. Two discrete dichotomous variables are used in the classification system. In the current study 60 cases were classified on the basis of histopathology and the same set of patients were subjected to immunohistochemistry and classified under molecular classification. The difference between the two classification systems and 95% confidence interval were determined. The p value was derived and its significance was studied. Inter rater agreement is used to evaluate the strength of agreement between two classification systems.

The agreement is quantified by the KAPPA statistic.

1. KAPPA is 1; when the agreement between the two classification systems is perfect.
2. KAPPA is 0; when there is no agreement better than chance.
3. KAPPA is negative; when agreement is worse than chance.

The Standard errors reported by MedCalc are the appropriate standard errors for testing the hypothesis that the underlying value of weighted kappa is equal to a pre-specified value other than zero. (Table4) The value of KAPPA, with its standard error and 95% confidence interval was derived. The agreement between the two classification systems was analyzed.

Table 4: Kappa value and its related strength of agreement

K value	Strength of agreement
<0.2	Poor
0.21-0.4	Fair
0.41-0.6	Moderate
0.61-0.8	Good
0.81-1.0	Very good

OBSERVATION AND RESULTS

In the study period of 29 months from January 2015 to June 2018, total number of breast specimens received were 1412 cases, of these breast tumours accounted for 1023 cases. The total number of non-neoplastic, benign and malignant cases was 289 (20.46%), 472(33.42%) and 651(46.11%) respectively. Among these malignant cases a total of 60 cases were subjected to ER, PR, HER2, CK5/6 and Ki67 and scoring was given. Based on which they were classified into luminal A, luminal B, HER 2, basal and unclassified as per molecular classification of which Luminal A constituted the most common type (36.7%), followed by 14 cases of basal type(23.3%), 8 cases of HER2 (13.3%), 6 cases of unclassified (10%) and 5 cases of luminal B and hybrid types(8.3%).

The clinicopathological parameters were compared with the molecular classification. The most common age groups affected by breast cancers are 50-59 years. Among the molecular classification, the luminal A and luminal B showed higher incidence of breast cancer in 50-59 age group with 40% incidence. The youngest age of presentation is at 26 years and oldest was 75 years old. On analyzing the side and site of involvement it was found that left sided upper outer quadrant tumors were more common in luminal A, luminal B, HER 2 and basal types with 55%, 60%, 82% and 57% respectively. 68% of the cases had a tumor size of 2-5 cm followed by tumors of greater than 5 cm size.

On analyzing the molecular classification and its comparison with the histological types it was found that among the 30 cases of infiltrating ductal carcinoma NST, 6 were luminal A and HER2 type, 3 were luminal B, 8 were basal, 5 were unclassified. 2 cases showed strong positivity of both HER2 and luminal markers and were considered as hybrid cases. Among the 30 cases of special variants 16 cases were luminal A, 2 case were luminal B and HER2, 6 were basal and 1 unclassified. 3 cases were found to express hybrid markers. Luminal A &B types had low histological grade and her2 and basal type had grade III of bloom Richardson grading system. Among all the molecular classification luminal tumors were less associated with adverse additional finding like lymphovascular invasion, perineural invasion, necrosis and skin involvement whereas Her2, basal and unclassified types showed 50%, 67% and 93% respectively.

Based on the hormone receptor, her2 and basal markers scoring all invasive breast carcinoma special and no special types were classified into luminal A&B, Her2 type and basal type. Some triple positive tumors were identified and classified as hybrid tumors. Few cases which didn't express any markers were classified under undifferentiated type. High proliferation index was seen in all Luminal B cases and 86% of basal cases. 90% Luminal A cases showed low proliferation index. All cases were followed up for a period of 6 months and found that 78% of cases were alive and healthy 17% had recurrent disease and 5 % were dead. Mortality was seen to be more among Her2, Hybrid and unclassified types with one case in each.

MC NEMAR'S TEST AND INTER RATER AGREEMENT:

The aim of this test is to evaluate the inter rater agreement between the histopathological and molecular classification systems and to quantitate the agreement with the KAPPA value by using the 2x2 classification tables. Each subtype under the molecular classification is compared with histopathological classification which is divided as infiltrating ductal carcinoma no special types and special variants by using the 2x2 tables. P value and the inter rater agreement KAPPA value is derived and analyzed. A negative KAPPA value indicates that the strength of agreement between these two classification systems is very poor. Negative agreement between these two classification systems helps in substantiating the use of targeted therapy based on molecular classification.

When Luminal A subtype was compared with the histological subtypes it was found that the P value was 0.2 and the KAPPA value was-0.367, which indicates that strength of agreement between these two systems was very poor and the agreement between them is worse than chance. Similarly basal type, Her2, Hybrid type and undifferentiated type had a significant P value of <0.0001 and the inter rater agreement KAPPA value was -0.033, -0.200 -0.033 and -0.034 respectively (Table 5). This negative value proves the disagreement between these two systems and agreement if any, is worse than chance and hence the importance of molecular classification is substantiated for the targeted therapy.

TABLE 5: Comparison of Molecular classification with histopathological classification

	MOLECULAR CLASSIFICATION					
HPE CLASSIFICATION	Luminal A	Luminal B	Her 2	Hybrid	Basal	Un classified
IDC NST	5	2	6	2	9	5
VARIANTS	17	3	2	3	5	1
Total	22	5	8	5	14	6
95% CI	7.51 to 35.26%	25.05% to 49.56%	28.6 %to 40%	25.05 %to 49.56 %	4.79% to 36.69%	23.14% to 44. 18%
Significance Level	**P=0. 2115**	**P < 0.0001**	**P<0. 0001**	**P<0. 0001**	**P= 0.0140**	**P <0.0001**
Inter rater agreement-KAPPA value	**-0.367**	**-0.0333**	**-0.02 00**	**-0.03 33**	**-0.0346**	-0.100

DISCUSSION AND CONCLUSION

Breast carcinoma is one of the most commonly diagnosed cancers in females worldwide comprising 16% of all female cancer cases. Study of tumor molecular characteristics has enhanced our understanding of both the tumor behaviour and the response to therapy. In this study of 60 cases which included invasive ductal carcinoma NST and its variants, an attempt has been made to evaluate the hormonal status and proliferation index by immunohistochemistry as per ASCOCAP guidelines.

Most of the findings were concurrent with the other studies for example 86% of mucinous tumors were hormone receptor positive which was concurrent with Lacorix-Triki et al., study (2010). 76% of medulary carcinoma were triple negative which was concurrent with Jensen et al study. [2011] All five cases of papillary and apocrine carcinomas were luminal A and were concurrent with Lotan et al [2009] and Matsuo et al., studies (2002).

Current study differed from literature in metaplastic carcinoma cases being hormone receptor positive and one case being her 2 positive (Tse et al., 2006). In this study, 27 cases of luminal tumours were graded as 11% of grade I, 22% of grade II and 5% of grade III tumour. After six months of follow up period of these tumours there was an increased incidence of recurrence rate reported in grade III tumors. Also this study shows that the HER2 and basal types had more number of grade III tumors. This was in concurrence with the study done by Rakha et al.

According to cheang et al [2008], they have concluded that the expanded immunopanel of five markers which composed of ER, PR, HER2neu, EGFR, and CK 5/6 than the usual triple biomarkers of ER, PR and HER2neu provides a better definition of basal like tumours and its disease free survival more specifically. They have found that not all the triple negative tumors express the basal markers. Only 9% out of 17% of triple negative tumors expressed basal markers. This similar finding was found in the current study, in which among 33% of triple negative tumors only 23% showed positivity for basal markers. The significance of demonstrating the basal type tumours is that they may benefit from EGFR targeted therapy and specified chemotherapy. Under the umbrella of triple negative tumours which has an overall poor survival and early recurrence, there are tumours with good prognosis as adenoid cystic carcinoma and secretory carcinoma. So it is essential to subtype triple negative tumours with basal markers. This is concluded in this study by constantindou et al [2010].

Luminal B and hybrid types had an increased incidence of recurrence in the study of Maggie cheang et al [2008]. In their study of immunohistochemical analysis of breast cancers have concluded that all the luminal B and luminal -HER 2 hybrid tumours were associated with poor recurrence free survival and disease survival, those who were treated with adjuvant systemic therapy. But in the current study, after the six month follow up period it was found that all he luminal B tumors were alive and healthy, whereas the luminal-HER2 hybrid and basal types had only 60% of cases with disease free survival. Therefore it is essential to identify the basal tumours and hybrid categories so that they are provided with additional therapies.

Treatment for breast cancers, given based on the histopathological classification is broad based and includes endocrine therapy, systemic chemotherapy and Herceptin therapy. Whereas when breast cancers were classified under molecular classification a better targeted therapy is provided, avoiding unnecessary drugs to patients who do not need it, thereby preventing unnecessary drug related toxicities and reducing the costs of the treatment. There exist a difference in the treatment options between the histopathological classification and molecular classification. Therefore it is necessary to prove the disagreement between these two classification systems. In this current study, each molecular subtype was compared with the histopathological classification and it was found that all the subtypes had a disagreement with the histopathological classification that was proved by the negative inter rater agreement KAPPA value. Disagreement between the two systems substantiates the value of molecular classification in the field of targeted therapy.

In the current study the treatment was given for some cases based on the histopathological classification and for some based on the immunohistochemical analysis of triple markers. In other studies of Hess KR et al [2006], Ayers M et al [2004], Gianni L et al[2005], they have found a significant reduction in the incidence of relapse, when treatment was targeted therapy based on molecular classification.

LIMITATIONS OF THE STUDY

1. The cases were selected on the basis of histopathological classification in the tertiary care centre and not a population base study, which will not reflect the true prevalence of the general population

2. Her 2 neu expression has an intermediate stain scoring of 2+ which requires FISH for grading it as negative or positive.

3. Gene expression profiling will give more accurate molecular subtypes than immunohistochemistry, but being expensive it cannot be applied to all patients.

4. Being a retrospective study the targeted therapy according to molecular classification was not given and hence the prognostic inference could not be ascertained.

To conclude, breast cancers are heterogeneous and having diverse clinical outcomes, these researches on molecular subgroups would pave way towards the "personalization" of treatment for breast cancers with the more feasible and economic tool of immunohistochemistry.

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