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Research Article

Breast Cancer: Correlation of Molecular Classification with Clinicohistopathology

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Abstract: Breast cancer is the most common cancer among women both in developed and developing countries. The current study was conducted to correlate the molecular sub-typing of breast cancer with the Histological typing, Bloom Richardson grading and TNM staging. A cross sectional study was conducted on 70 invasive breast carcinoma samples from November 2012 to October 2014. Cases were sub-classified using immunohistochemical staining for ER, PR, Her-2/neu, Ki-67, CK 5/6 into following subtypes luminal A, luminal B, Her-2/neu positive, basal-like and normal-like. Most common molecular subtype was Luminal A representing 27.1% of total cases, and least common subtype was normal-like comprising 5.7% of total cases. Luminal B, Her-2/ neu positive, Basal-like molecular subtypes belonged to 25.7%, 25.7%, 15.7% cases respectively. The following variables were significantly associated with IHC subtypes: number of lymph node involved, Bloom-Richardson grading and TNM staging with p value < 0.05. Luminal B, Her-2/neu positive and basal-like phenotype are found to be more associated with Large tumor size, high Nottingham modification of Bloom- Richardson Grade and high TNM stage than with luminal A and normal-like phenotype.
Keywords: Breast cancer, Bloom Richardson Grading, CK 5/6, ER, Immunohistochemistry, Her-2/neu.

INTRODUCTION

Breast cancer is the most common cancer among women both in developed and developing countries. It ranks as the fifth most common cause of death from cancer overall. Incidence of breast cancer in India is lower as compared to the west. In our country, it is the 2nd leading cause of cancer among women's preceded by cervical cancer [1].

Breast cancer is characterized by cellular heterogenicity. Breast tumors with similar histopathological appearances can exhibit divergent clinical presentations, disease aggressiveness and treatment responsiveness. These differences are possibly due to the limitation of the current classification of breast cancers, based mainly on morphology. Analysis of gene expression profiling and immunophenotypic characteristics suggests that breast cancer is not a single entity but a heterogeneous disease [2-4]. Breast cancers were categorized into at least five main groups which differ markedly in terms of distinct races/ethnicities, risk factors distribution, prognosis, therapeutic treatment responsiveness, clinical outcomes and both overall survival (OS) and relapse-free survival: luminal cell-like tumors, subdivided into luminal A and B , Her-2/neu positive, basal -like (BCL), and normal breast-like group [5-7]. Luminal A tumors were shown to be associated with good prognosis and a less aggressive behavior if compared with the BCL or Her-2/neu groups.5 Basal-like subtypes has been associated with aggressive behavior, poor clinical outcomes, lack of response to the usual endocrine therapies, shorter survival and BRCA1 mutations [8, 9]. Several studies have shown that breast carcinomas may be stratified in subtypes similar to those defined by expression profiling using a panel of immunohistochemical (IHC) markers [10]. Routine IHC evaluations of breast cancers may therefore provide not only crucial information to guide clinical management

but also represents a valid alternative to costly genotyping assays.

In the present study, we therefore investigated prevalence and correlate the clinicopathological features of breast cancer patients classified according to molecular subtypes as defined by a restricted panel of IHC markers.

MATERIALS AND METHODS

Sampling Frame

A cross sectional study was conducted on 70 invasive breast cancer samples diagnosed in Department of Pathology from November 2012 to October 2014. 70 samples were collected as these 70 were the total number of breast cancer samples during the total study duration of 2 years.

Immunohistochemistry

Firstly cut 3-4 micrometer thick formalin - fixed paraffin embedded tumor section and gently lowered on surface of water bath at 45˚ C and spread wrinkle free on to the poly L-lysine coaled slide. After deparrafinization in Xylene, slides were rehydrated through graded series of alcohol and placed in phosphate buffer. Antigen retrieval was done in pressure cooker by using citrate buffer. Endogenous peroxide activity is blocked by peroxide block. Commercially available monoclonal antibodies to ER, PR, Her-2/neu, Ki-67, CK 5/6 W were used in this study for antigen localization, than add secondary antibody (polymer Horse radish peroxidase). 3,3'-diaminobenzidine tetrahydrochloride(DAB) chromogen was used for colour production. Positive and Negative control were stained with each IHC staining batch. Each case was stain for ER, PR, Her-2/neu, Ki-67 and CK 5/6 then sub-classified into luminal A, luminal B, Her-2/neu positive, basal-like and normal-like molecular subtypes (Table 1).

Reporting parameter and interpretation of results

The ER and PR results were screened manually and interpreted as positive when more than 10% of neoplastic cells were positive as done in previous studies [11-13]. The known cases of carcinoma breast served as positive control. The following method was used to score Her-2/neu over expression: cases showing no membrane immunostaining or membrane staining was observed in less than 10% of the tumor cells were scored 0, cases with faint/barely perceptible and incomplete membrane staining in more than 10% of the tumor cells were scored 1+, cases with weak to moderate complete membrane staining in more than 10% of the tumor cells were score 2+,and at last cases with strong complete membrane staining in more than 10% of the tumor cells were score 3+. A score of 3+ were considered positive. In each case, negative benign breast condition was used as internal controls and invasive breast carcinoma case with known Her-2/neu over expression was used as a positive control for each batch. Ki-67 was reported high if >14% neoplastic cells were showing Nuclear immunoreactivity. Germinal center of lymph node used as positive control. Cytokeratin 5/6 were reported positive if 11-100% tumor cells showing Cytoplasmic immunoreactivity . Epidermal layer of skin considered as positive control.

Statistical analysis

Age difference was examined using One way analysis of variance (ANOVA). X2 (chi-square) was used to draw the correlation between various immunological markers. Fisher exact test was applied to compare the following variables: tumor grade, tumor size, tumor stage and histological type. Odds ratio (OR) and 95% confidence intervals (CI) were also applied to estimate the magnitude of association among various immunological markers. P value less than 0.05 was considered significant.

RESULTS

A total of 70 cases of infiltrating breast carcinoma were included in this study. 37.2% cases belonged to age-group of 41-50 years with mean age of 47.76 years**.** There was only one male case (1.4%) and rest 69 were female cases (98.6%). In 48 (68.6%) cases left breast was involved, right breast involvement seen in 22 (31.4%) cases. 63(90 %) cases were diagnosed as Invasive Ductal carcinoma, 3 cases of lobular and 2 each of medullary and mucinous type respectively. In Bloom Richardson grading 60% cases belongs to grade II, 22.9% grade III and 17.1% to grade I. 61.4% cases had tumor size more than 5 cm (T_3), 37.2% had tumor size 2 to 5 cm (T_2) and only 1.4% cases had tumor size between 1 to 2cm (T_1). According to TNM staging, 57.2% cases belonged to Stage III, 41.4 % to Stage II and only 1.4% cases belonged to Stage I. In T_1, none of the cases had lymph node involvement, while in T_2 and T_3, 38.5% and 61.5% cases had positive lymph node status respectively.

The proportion of various molecular subtypes was as follows: luminal A 27.1%, luminal B 25.7%, Her-2/neu positive 25.7%, basal-like 15.7% and normal-like 5.7%. Her-2/neu positive and basal-like subtype are associated with more number of lymph node involvement ($p<0.05$), as compare to luminal A, luminal B and normal-like subtype. Luminal B,Her-2/neu positive and basal-like subtype are associated with higher Bloom Richardson grade ($p<0.05$)and advanced TNM stage at the time of diagnosis($p<0.05$) , as compare to luminal A and normal-like subtype.

In the all molecular sub types, most common histological type was Invasive ductal carcinoma. In invasive ductal carcinoma most common molecular subtype was luminal A and luminal B with each comprises of 26.9% cases, followed by Her-2/neu, basal-type, normal-like subtypes 23.8%, 15.8%, 6.3% respectively (Table 2).

Table 1: Antibodies used in study with their specification

Antibody	Dilution	Staining pattern	Company
Anti-Estrogen receptor	Ready to use	Nuclear	Biogenex.
Anti-Progesterone receptor	Ready to use	Nuclear	Biogenex
Anti-Her-2	Ready to use	Membrane	Biogenex.
Anti-Ki-67	Ready to use	Nuclear	Biogenex
Anti-CK 5/6	Ready to use	Cytoplasmic	Biogenex

Table 2: Clinopathological and Immunohistochemical characteristics of Breast cancer cases

Variables		All cases (n-70) 100%	Luminal A (n-19) 27.1%	Luminal B (n-18) 25.7%	Her-2/neu Positive (n-18) 25.7%	Basal-like (n-11) 15.7%	Normal-like (n-4) 5.7%	p value
Age	Mean	47.76	49.63	48.22	46.50	49.09	38.75	**0.471**
Laterality	Left	48(68.6)	14(73.7)	14(77.8)	10(55.6)	7(63.6)	3(75)	0.641
	Right	22(31.4)	5(26.3)	4(22.2)	8(44.4)	4(36.4)	1(25)	
B-R grade	Grade l	12(17.1)	4(21.1)	1(5.6)	3(16.7)	1(9.1)	3(75)	0.016*
	Grade ll	42(60.0)	13(68.4)	15(83.3)	8(44.4)	5(45.5)	1(25)	(<0.05)
	Grade ll	16(22.9)	2(10.5)	2(11.1)	7(38.9)	5(45.5)	0	
Tumor size	T_1	1(1.4)	1(5.3)	0	0	0	0	0.692
	T_2	26(37.2)	9(47.4)	7(38.9)	5(27.8)	3(27.3)	2(50)	
	T_3	43(61.4)	9(47.4)	11(61.1)	13(72.2)	8(72.7)	2(50)	
Lymph node involved	Negative	18(25.7)	6(31.6)	4(22.2)	7(38.9)	1(9.1)	0	0.408
	Positive	52(74.3)	13(68.4)	14(77.8)	11(61.1)	10(90.9)	4(100)	
ER	Positive	36(51.4)	18 (94.7)	18 (100)	0	0	0	<
	Negative	34(48.6)	1 (5.3)	0	18 (100)	11 (100)	4 (100)	0.001*
PR	Positive	33(47.1)	17 (89.5)	16 (88.9)	0	0	0	<
	Negative	37(52.9)	2 (10.5)	2 (11.1)	18 (100)	11 (100)	4 (100)	0.001*
Her-2/neu	Positive	36(51.4)	0	18(100)	18(100)	0	0	< 0.001
	Negative	34(48.6)	19 (100)	0	0	11 (100)	4 (100)	*
Ki-67	Positive	16(22.9)	6 (33.3)	3 (16.7)	4 (36.4)	0	6 (33.3)	0.426
	Negative	54(77.1)	12 (66.7)	15 (83.3)	7 (63.6)	4 (100)	12 (66.7)	
CK 5/6	Positive	15(21.4)	1 (5.3)	2 (11.1)	1 (5.6)	11 (100)	0	<
	Negative	55(78.6)	18(94.7)	16(88.9)	17 (94.4)	0	4 (100)	0.001*

*Statistically Significant

Table 3: Proportion of different molecular subtypes found in different studies

	SUBTYPE OF BREAST CANCER				
Author	**Luminal A**	**Luminal B**	**Her 2/neu**	**Basal-type**	**Normal-like**
Lisa A. Carey *et al.* [6]	51.6%	15.5%	6.6%	20.1%	6.2%
Livasy *et al.* [27]	61.0%	23.0%	16.0%	8.0%	6.0%
Fan *et al.* [20]	42.0%	19.0%	12.0%	18.0%	9.8%
Yang *et al.* [28]	69.0%	6.0%	8.0%	12.0%	6.0%
C. A. Adebamowo *et al.* [18]	77.6%	2.6%	4.0%	15.8%	----
D. Huo *et al.* [17]	27.0%	2.0%	15.0%	27.0%	28.0%
Spitale A *et al.* [19]	73.0%	13.0%	5.6%	7.4%	-----
Munjal K *et al.* [11]	37.0%	11.0%	29.0%	7.5%	15.0%
A. Sherif *et al.* [26]	59.1%	16.4%	12.7%	11.8%	----
B. S. Coya *et al.* [21]	44.0%	26.6%	11.8%	11.3%	7.9%
Yinghao Su *et al.* [23]	48.6%	16.7%	13.7%	12.9%	-----
El-Hawary AK *et al.* [29]	41.2%	13.9%	19.4%	28.5%	-----
Asmerom Tesfamariam *et al.* [22]	55.0%	5.0%	5.0%	10.0%	25.0%
Present study	27.1%	25.7%	25.7%	15.7%	5.7%

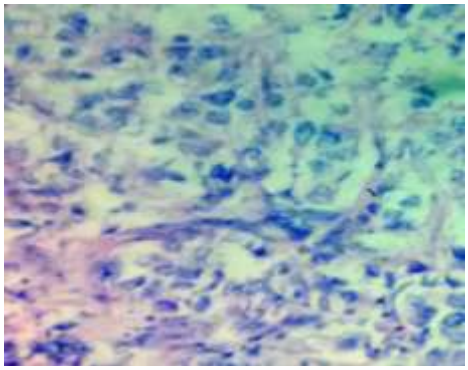

Fig. 1: Luminal A: Infiltrating duct carcinoma Nottingham modification of Bloom-Richardson's Grade –II, H & E stain slide- 40X

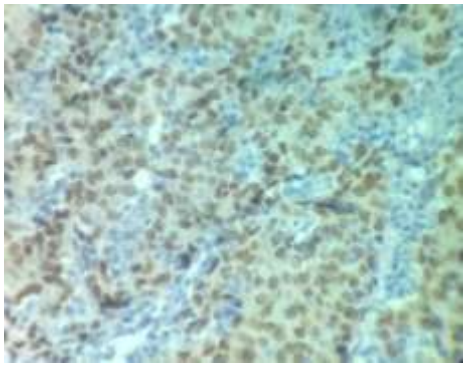

Fig. 2: Luminal A- IHC stain slide for estrogen receptor Quick Score 8/8 -40X

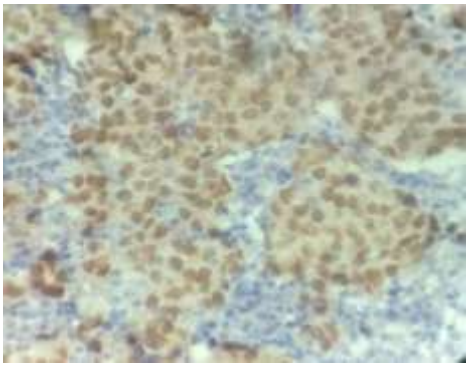

Fig. 3: Luminal A- stain slide for progesterone receptor Quick Score 8/8 - 40X

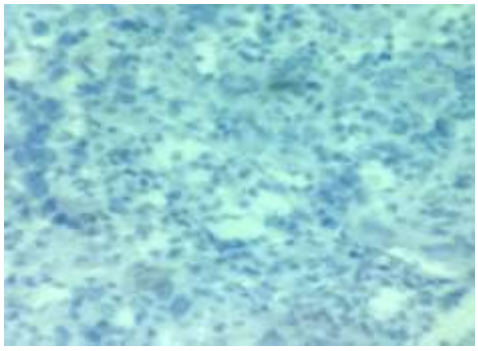

Fig. 4: Luminal A- IHC stain slide for HER-2/neu receptor showing negative staining -40X

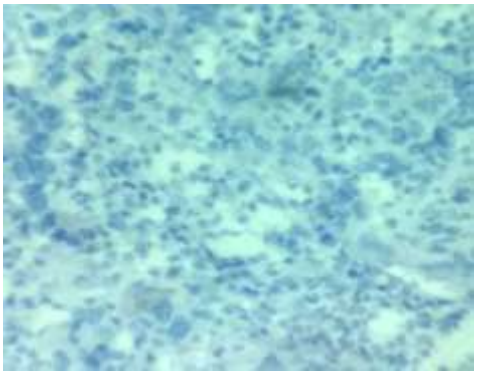

Fig. 5: Luminal A- IHC stain slide for Ki-67 receptor showing negative staining -40X

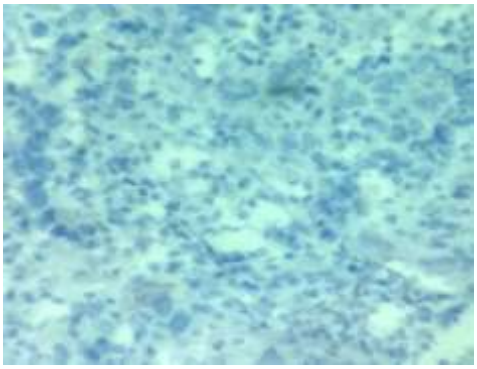

Fig. 6: Luminal A- IHC stain slide for CK 5 & 6 receptor showing negative staining -40X

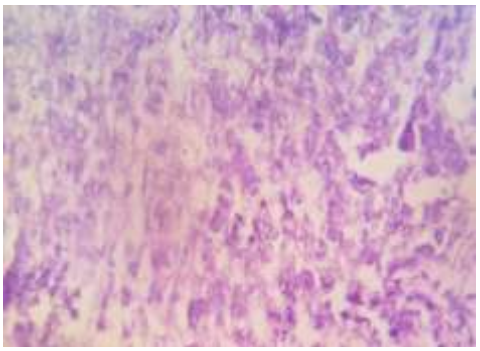

Fig. 7: Luminal B-Infiltrating duct carcinoma Nottingham modification of Bloom-Richardson's Grade –II, H & E stain slide- 40X

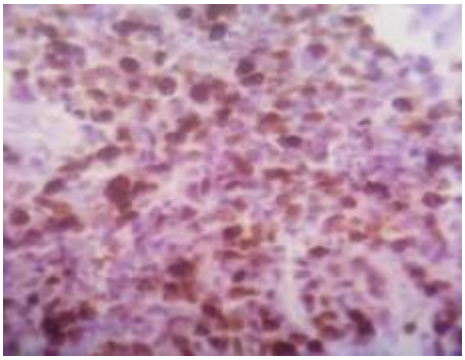

Fig. 8: Luminal B- IHC stain slide for estrogen receptor Quick Score 8/8 -40X

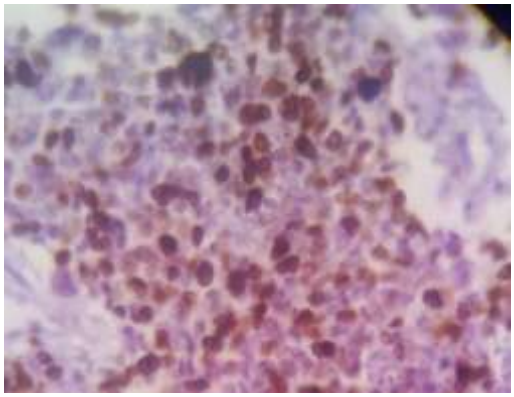

Fig. 9: Luminal B- IHC stain slide for progesterone receptor Quick score 8/8 -40X

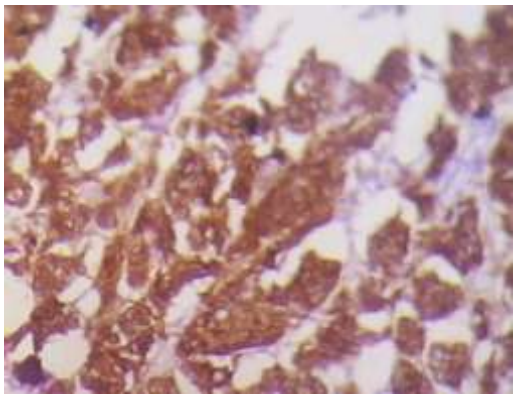

Fig. 10: Luminal B- IHC stain slide for HER-2/neu showing diffuse membranous staining – score 3+ -40X

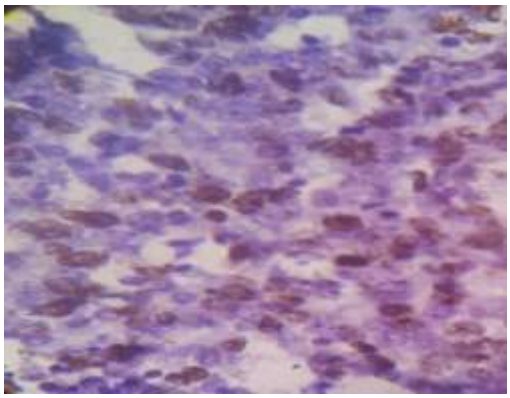

Fig. 11: Luminal B- IHC stain slide for Ki-67 receptor showing high score -40X

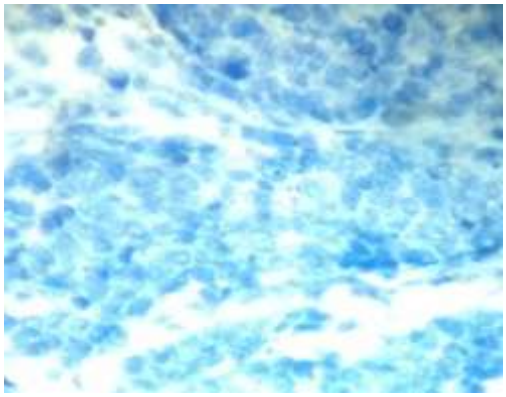

Fig. 12: Luminal B- IHC stain slide for CK 5 & 6 receptor showing negative staining -40X

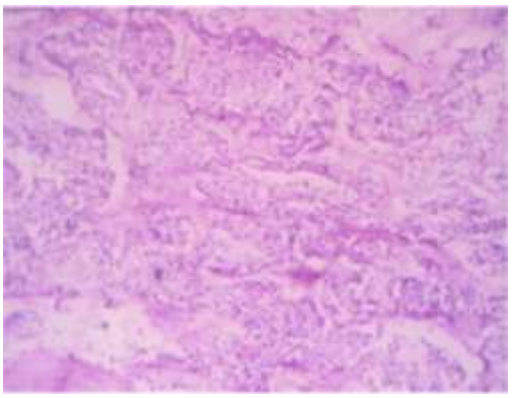

Fig. 13: HER- 2/neu positive- Infiltrating duct carcinoma Nottingham modification of Bloom-Richardson's Grade –III H & E Stain Slide- 40X

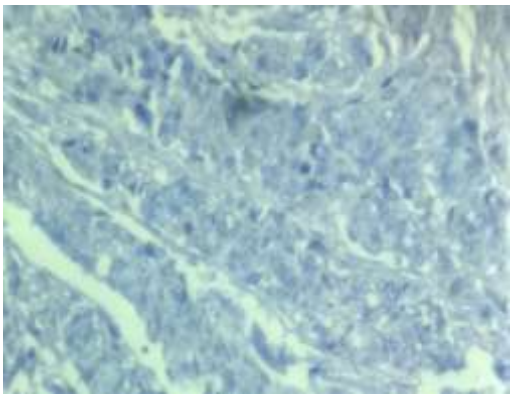

Fig. 14: HER- 2/neu positive- IHC stain slide for estrogen receptor showing negative staining - 40X

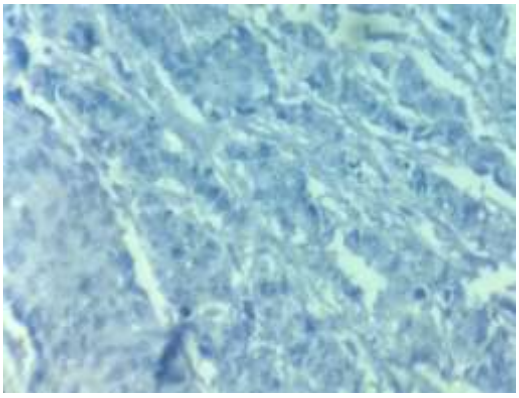

Fig. 15: HER- 2/neu positive- IHC stain slide for progesterone receptor showing negative staining -40X

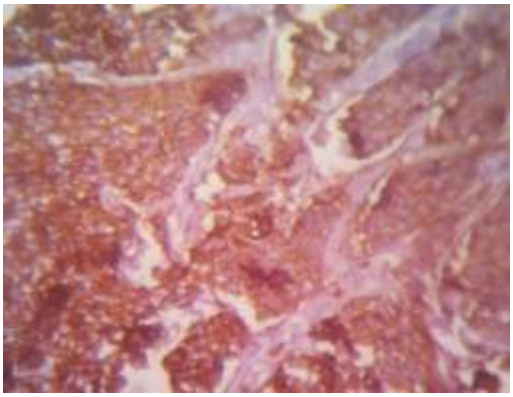

Fig. 16: HER- 2/neu positive- IHC stain slide for HER-2/neu showing diffuse membranous staining –score 3+ -40X

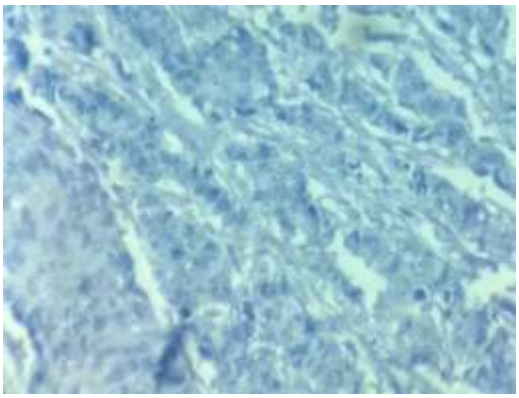

Fig. 17: HER- 2/neu positive- IHC stain slide for Ki-67 receptor showing low score-40X

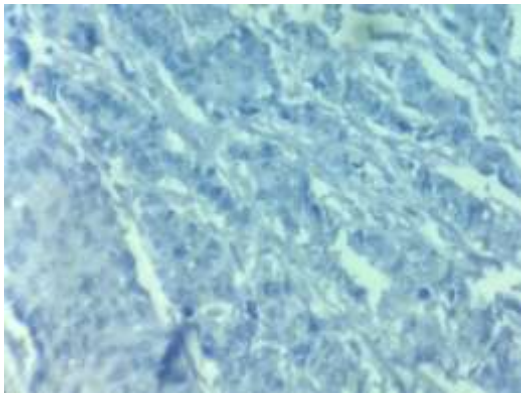

Fig. 18: HER- 2/neu positive- IHC stain slide for CK 5 & 6 receptor showing negative staining -40X

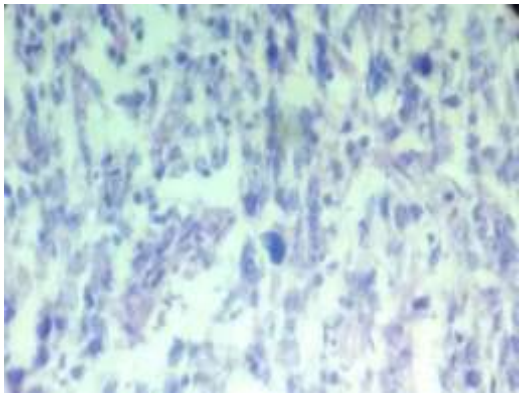

Fig. 19: Basal like- Infiltrating duct carcinoma Nottingham modification of Bloom-Richardson's Grade –III, H & E stain slide-40X

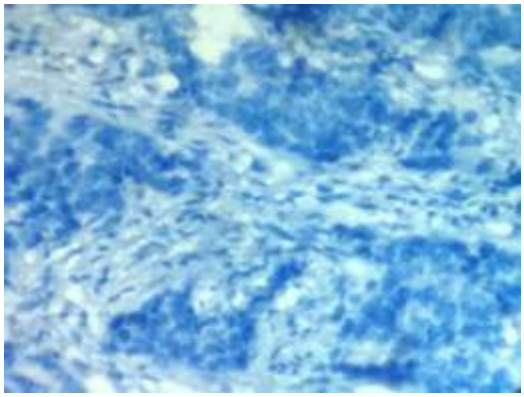

Fig. 20: Basal like- IHC stain slide for estrogen receptor showing negative staining -40X

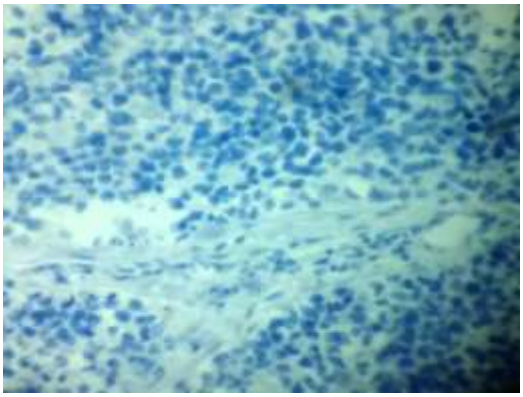

Fig. 21: Basal like- IHC stain slide for progesterone receptor showing negative staining -40X

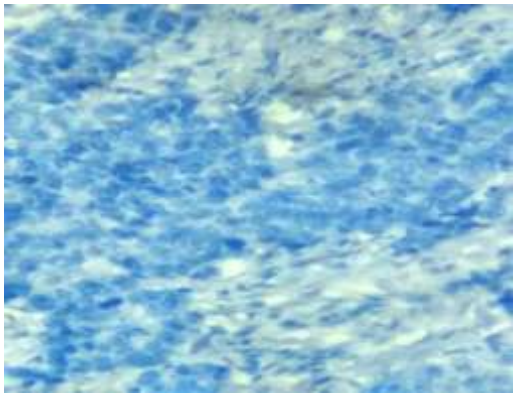

Fig. 22: Basal like- IHC stain slide for HER-2/neu receptor showing negative staining -40X

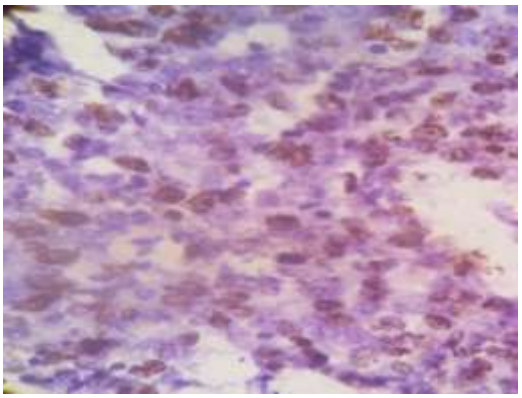

Fig. 23: Basal like- IHC stain slide for Ki-67 receptor showing high score-40X

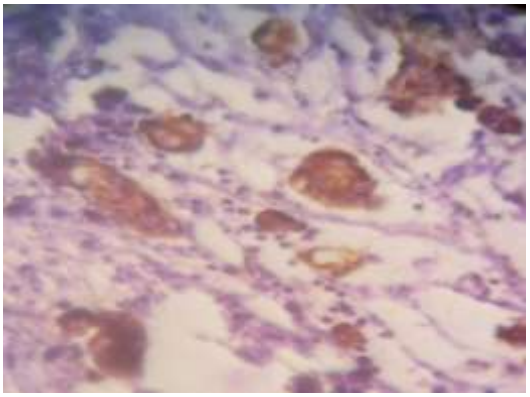

Fig. 24: Basal like- IHC stain slide for CK 5 & 6 receptor showing positive staining -40X

DISCUSSION

This study comprised 70 cases of invasive breast carcinoma with mean age at the time of diagnosis was 47.76 ± 11.08 years. Maximum incidence of breast cancer (37.1%) was observed in the age-group of 41-50years.In India, the average age of developing breast cancer has shifted over the last few decades and younger women (40-50 yr) are being affected. The lifestyle factors such as late age at marriage, reduced breast feeding, and westernization of diet may be associated with occurrence of breast cancer in younger population in India [14]. Most of cases (98.6%) were females, only one case (1.4%) of male breast cancer, this is comparable to Shet T *et al.* [15] who found 1.6% of male breast cancer in total breast cancer cases. 68.6% cases had left side breast cancer which is similar to study by Moses Ambroise *et al.*[13] who reported 59.2% cases in left breast.

In developed countries, in majority of patients lymph node was not involved, but studies carried out in India documented a greater percentage of breast carcinoma with lymph nodal metastasis compared to western figures [12]. In this study total cases with lymph node involvement was 74.3%, same were observed by Munjal K *et al.* [11] and Moses Ambroise *et al.*[13]. Most common histological type is invasive ductal carcinoma (NOS) comprises 90% of total cases, which is similar to other Indian studies [11, 13].

60% cases belongs to grade II of Bloom Richardson grading, while 22.9% and 17.1% cases belonged to grade III and grade I respectively, which is comparable to study done by Moses Ambroise *et al.* [13]. 57.2% cases belongs to Stage III , 41.4 % cases to Stage II and only 1.4% cases belongs to Stage I, same were reported by Chopra B *et al.* [14]. Literature shows that in India majority of new cases are advanced or locally advanced or higher stage at the time of diagnosis. It was observed that, 19.3 % of cases with positive lymph node status belonged to grade I, whereas 57.7% and 23.0% cases with positive lymph node status belonged to grade II and grade III

respectively, Amrut V *et al*. [16] found almost similar results.

In this study most common molecular subtype was luminal A representing 27.1%, which is compatible with study done by D. Huo *et al.* [17], however other studies done in west and in India reported high figure [18, 19, 11]. Least common molecular subtype was normal-like, which is similar to study done by Fan *et al.* [20] and B. S. Coya *et al*. [21]. However frequency of normal-like according to the study done by D. Huo *et al*. [17], Munjal K *et al.* [11, Asmerom Tesfamariam *et al*. [22] was 28% , 15%, 25% respectively. We observed a higher proportion of Her-2/neu subtype (25.7%) than previously reported in literature, same were observed by Munjal K *et al*.[11] (29.0%).

There was no correlation found between molecular type of breast cancer and age of the patients. (p value = 0.948), However study done by Munjal K *et al*. [11] and Yinghao Su *et al*. [23] signifies that, luminal A subtype found in slightly older women. Luminal B, Her 2/neu and basal-type subtypes associated with large tumor size than luminal A subtypes. However this finding does not reach any statistically significant value, this is similar to study done by Brenton JD *et al.* [24], Munjal K *et al.* [11].

Majority of cases of luminal A, luminal B and normal-like subtypes had less number of lymph node (1-3) involvement (42.1%,38.9% ,100% cases respectively) while in Her-2/neu and basal-like phenotype majority of cases had more number of lymph node (4-9) involvement (50%,63.3% respectively). This association was found to be statistically significant with p value = 0.015. Present study corresponded to study done by Kim *et al.* [25], Spitale A *et al.* [19], Munjal K *et al.* [11] and A. Sherif *et al.* [26].

Luminal A had lower tumor grade while Her-2/neu positive and basal-type phenotype are associated with higher grade tumors. This association was found to be statistically significant with p value = 0.016. This finding was comparable to study done by Spitale A *et al*. [19], Munjal K *et al.*[11] and A. Sherif *et al*. [26].

Luminal B, Her-2/neu and basal-like are associated with higher stage than luminal A, which are associated with earlier stage. This study Statistically found to be Significant with p value = 0.037.This is similar to studied by Spitale A *et al*. [19], Munjal K *et al*. [11] and Yinghao Su *et al*. [23] (Table 3).

CONCLUSION

Carcinoma of breast is a common clinical problem in our society. Patients usually present in late stage mainly due to lack of diagnosis at an early stage. There is an immediate need for breast cancer screening, health education and public awareness programs (including self-palpation of breast) to detect the disease in early stages. Present study showed that large tumor size, high Nottingham modification of Bloom-Richardson Grade and high TNM stage are usually associated with Luminal B, Her-2/neu positive and basal-like phenotype than luminal A and normal-like phenotype. Luminal B, Her2/neu positive and basal-like is associated with poor prognosis than luminal A, and normal-like phenotype. As the traditional histological classification are not able to evaluate the biological behavior of the different breast tumors, molecular classification of breast cancer is useful for clinical management and superior to the histological classification in short term prognostic value. Different immunophenotypes respond differently to different therapies. Luminal groups respond to hormonal treatment while her-2/ neu group respond well to biological therapies using transtuzumab. On the other hand, basal like phenotype, usually respond well to chemotherapy. In the light of above findings and the availability of newer drugs, hormonal therapy and biological therapies, this type of classification must be investigated and taken into account when assessing response to these treatments.

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