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Original Research Article

Immunohistochemical Characterisation of Basal Like Phenotype of Triple Negative Breast Cancer from Western India

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ABSTRACT

Background and Aim: Triple-negative breast cancer (TNBC), which accounts for approximately 15-20% of all breast cancers, defined as lack of expression of estrogen receptor, progesterone receptor and Her-2 neu receptors. TNBC has two subtypes basal like and non-basal like, the former characterised by aggressive biology with limited therapeutic options. The present study aimed to identify TNBC with basal like phenotype and its correlation with conventional parameters.
Method: This study enrolled 100 untreated TNBC and immunohistochemical localisation of CK5/6, CK14, CK17, EGFR, Vimentin and C-kit was performed on tumor tissues for identification of basal like phenotype. The data was analysed statistically using SPSS software version 20.
Results: CK5/6, CK14, CK17, EGFR, Vimentin and C-kit expression was seen in 36%, 4%, 45%, 45%, 50% and 38% TNBC, respectively. Of 100 TNBC patients, 77% were positive for basal marker expression identified as basal like and 23% were negative for basal marker expression identified as Non-Basal. Further, multivariate analysis by General Linear Model indicated that four markers combination (vimentin, EGFR, CK17, CK5/6) has maximum 1.00 observed power of the test for identification of Basal-like TNBCs. In univariate and multivariate survival analyses, none of the marker discriminated patients with worse or better relapse free survival.
Conclusion: A panel of markers includes Vimentin, EGFR, CK17 and CK5/6 can be used for classification of basal like TNBCs.

Keywords: TNBC, Basal like, Immunohistochemistry markers

INTRODUCTION

In India, breast cancer has been declared the most common form of cancer in women, almost surpassing cervical cancer. Breast cancer risk in India revealed that 1 in 28 women develop breast cancer during her lifetime. This is higher in urban areas being 1 in 22 in a lifetime due to change in life style factors compared to rural areas where this risk is relatively much lower being 1 in 60 women developing breast cancer in their lifetime. At Gujarat Cancer & Research Institute (GCRI), the incidence of breast cancer is 23% among female cancers as per hospital based registry.

Biologically, breast cancer is a very heterogeneous disease and molecular classification has become more powerful than histopathology as a predictive factor for treatment selection. Comprehensive gene-expression patterns of breast cancer by

DNA microarray suggested four major molecular classes of breast cancer are luminal-like, basal-like, normal-like, and HER-2 positive. DNA Microarray is very expensive and therefore immunohisto-chemistry technique is now routinely used in clinics to divide breast cancer into four major molecular subtypes such as Luminal A, Luminal B, Triple negative/basal-like, and HER2 type. Breast cancer prognosis and therapeutics are largely dependent on these subtypes. There are targeted therapies available for hormone receptor positive (ER+PR+) and her2 positive breast cancer however in triple negative breast cancer (TNBC) there is no such specific therapy available. It has been suggested that TNBC had a greater risk for systemic recurrence and death than did those with non-TNBC. These events occurred mostly within the first 5 years after diagnosis, as the risk for systemic recurrence for patients with TNBC peaked at 3 years and declined rapidly afterwards, whereas non-TNBC patients' risk for recurrence was constant. [1] Patients with TNBC have a higher rate of visceral metastasis and their tumors are more likely to harbor abnormalities in the p53 and BRCA1 genes compared with patients with non-TNBC. In TNBCs two subtypes defined by gene expression profiling are basal like and non basal like. In general, non basal TNBC has better prognosis. Basal like TNBC expresses basal markers like cytokeratin (CK) 5/6, CK17, Vimentin and EGFR. [2,3] Although the majority of cases of basal like breast cancer are triple-receptor-negative, 5% to 15% are not.

In view of this, the present study aimed to evaluate incidence of TNBC with basal like phenotype at Gujarat Cancer and Research Institute (GCRI) and its correlation with clinicopathological parameters and disease outcome.

MATERIALS AND METHODS

Patients

This retrospective study included 100 TNBC patients who had been diagnosed and treated at GCRI during 2014 to 2019 were included in the study. The detailed clinical history such as patient's age, menopausal status, disease stage, histopathological findings, treatment offered and disease status was recorded from the case file maintained at the Institutional Medical Record Department. Formalin fixed paraffin embedded tumor tissue (FFPE) blocks were collected from Histopathology department of the institute. Disease staging was done according to UICC TNM classification. Disease status was assessed by clinical examination, radiological investigations and biochemical investigations. The study was approved by Institutional Scientific Review Board and Ethics Committee. Patients other than TNBC subtype and subjected to neo-adjuvant therapy (either Radiotherapy or Chemotherapy before surgery) and patient with stage IV TNBC were excluded from the study.

Immunohistochemical localization

The 4µm thin sections were cut on microtome (Leica, Germany) and taken on 3-aminopropyl triethoxysilane (APES) coated slides. Immunohistochemical localization of CK 5/6, CK14, CK17, EGFR, Vimentin and C-kit was performed on FFPE tissue blocks containing primary tumor and evaluated by Haematoxylene and Eosin (H&E) staining, on Ventana Benchmark XT autoimmunostainer using Ventana reagents (Ventana, USA). Briefly, the protol includes following steps of deparaffinization using EZ solution, antigen retrieval using cell conditioning (CC1), incubation with ultra view DAB inhibitor for 4 minutes, 100µl of primary antibody, ultra view HRP multimer for 8 minutes, ultra view DAB detection kit for 8 minutes, counterstain with haematoxylin for 8 minutes, bluing reagent for 4 minutes and mounted with DPX. The primary antibody clone, company, dilution, antigen retrieval time and antibody dilution used are as follows:

Primary antibody	Clone	Company Name	Dilution	Cell Conditioning	Primary antibody incubation time (min)
CK5/6	D5&16B4	Cell Marque	1:100	Mild	32
CK14	EP162Y	BioGeneX	1:30	Mild	32
CK17	E207	BioGeneX	1:30	Mild	32
EGFR	EP38Y	Cell Marque	1:50	Mild	120
Vimentin	V9	BioGeneX	1:200	Mild	32
C-kit	EP10	BioGeneX	1:30	Standard	32

Scoring

Two independent observers familiar with Immunohistochemistry and unaware of the clinical outcome scored all the sections. The sections were scored with semiquantitative scoring ranging from negative (no staining) to 3+ (1+: staining in <10% of cells, 2+: staining in 10% to 50% of cells, and 3+: staining in >50% of cells) For statistical evaluation, scores 1+, 2+ and 3+ were taken together as positive group.

STATISTICAL ANALYSIS

Statistical analysis was carried out using SPSS statistical software version 20 (SPSS Inc, USA). Pearson's Chi-square test with Pearson's correlation coefficient (r) was used to assess correlation and significance between the two parameters. In case of patient number less than 5 in the cells of 2 x 2 tables, Yates' Continuity Correction value along with its significance was taken into consideration. Univariate survival analysis was carried out by Kaplan and Meier method and Log Rank statistics was used to assess the prognostic significance of disease free survival (DFS) and overall survival (OS). Multivariate survival analysis was performed using Cox regression model with forward stepwise (likelihood ratio) method. The Wald statistics and relative risk [Exp(B)] with 95% confidence interval (CI) for Exp(B) were used to evaluate the prognostic significance. ROC curve analysis was performed to evaluate significance of marker which

identify TNBC subtype. P values ≤ 0.05 were considered significant.

RESULTS

CK5/6 expression

In 100 TNBC patients, cytoplasmic expression of CK5/6 was noted in tumor tissue of 36% of the patients with an intensity of 1+, 2+ and 3+ in 19%, 10%, and 7% of the patients, respectively (Figure 1a).

In relation to clinicopathological parameters, CK5/6 expression was found similar in subgroups of age, menopausal status, lymph node status, histologic grade, and BR score. In relation to histological type, 36% patients with IDC exhibited CK5/6 expression (Table 1).

CK14 expression

Cytoplasmic expression of CK14 was noted in tumor tissue of 4% of the patients with an intensity of 1+, and 2+ in 1% and 3% of the patients, respectively (Figure 1b). Due to 4% positivity of CK14, further correlation with clinicopathological parameters was not done.

CK17 expression

Cytoplasmic expression of CK17 was noted in tumor tissue of 45% of the patients with an intensity of 1+, 2+ and 3+ in 5%, 18%, and 22% of the patients respectively (Figure 1c).

In relation to clinicopathological parameters, CK17 expression was found significantly higher in lymph node negative patients (p=0.05). Further, with advancement of disease stage the incidence of CK17 expression decreases (P=0.002). In relation to histological type, 50% patients with IDC exhibited CK17 expression (Table 1).

EGFR expression

Membranous expression of EGFR was noted in tumor tissue of 45% of the patients with an intensity of 1+, 2+ and 3+ in 2%, 20% and 23% of the patients, respectively (Figure 1d).

In relation to clinicopathological parameters, EGFR expression was found significantly high in histologic grade III tumors (P=0.001) and high BR score tumors (P=0.003) as compared to their respective

counterparts. A trend of low incidence of EGFR expression was noted in lymph node negative patients. In relation to histological type, 36% patients with IDC exhibited EGFR expression (Table 1).

Vimentin expression

Cytoplasmic expression of vimentin was noted in tumor tissue of 50% of the patients with an intensity of 1+, 2+ and 3+ in 7%, 13% and 30% of the patients, respectively (Figure 1e).

In relation to clinicopathological parameters, vimentin expression was found significantly high in histologic grade III tumors (P=0.05) and high BR score tumors (P=0.02) as compared to their respective counterparts. In relation to histological type, 46% patients with IDC exhibited vimentin expression (Table 1).

Table 1: Incidence of marker expression in triple negative breast cancer.

Parameter	N =100	CK5/6+	CK14+	CK17+	Vimentin +	EGFR+	Ckit+
Age <45 years	58	20(35)	03(05)	24(41)	29(50)	27(47)	19(33)
Age ≥45 years	42	16(38)	01(02)	21(50)	21(50)	18(43)	19(45)
Premenopausal	35	12(34)	01(03)	19(54)	19(54)	16(46)	10(29)
Postmenopausal	65	24(37)	03(05)	26(40)	31(48)	29(45)	28(43)
T1 size	08	04(50)	00(00)	05(63)	03(38)	02(25)	03(38)
T2 size	84	31(37)	04(05)	38(45)	42(50)	39(46)	34(41)
T3 size	08	01(13)	00(00)	02(25)	05(63)	04(50)	01(13)
LN Negative	62	23(37)	01(02)	33(53) [a]	31(50)	32(52)	26(42)
LN Positive	38	13(34)	03(08)	12(32) [a]	09(50)	13(34)	12(32)
Stage I	02	02(100)	00(00)	2(100) [b]	01(50)	01(50)	01(50)
Stage II	87	31(36)	04(05)	43(49) [b]	43(49)	39(45)	35(40)
Stage III	11	03(27)	00(00)	00(00) [b]	06(55)	05(46)	02(18)
HG I	07	03(42)	01(14)	03(43)	04(57)[c]	02(29)[d]	01(14)[e]
HG II	54	20(37)	03(06)	26(48)	21(39)[c]	16(30)[d]	16(30)[e]
HG III	39	13(33)	00(00)	16(41)	25(64)[c]	27(69 [d]	21(54)[e]
BR Score (N=85) Low	11	05(46)	01(09)	06(55)	04(36) [f]	02(18)[g]	04(36)
BR Score Intermediate	45	16(36)	03(07)	23(51)	19(42) [f]	16(36) [g]	13(29)
BR Score high	29	11(38)	00(00)	10(35)	21(72) [f]	20(69) [g]	15(52)
No Metastasis	85	30(35)	04(05)	38(45)	43(51)	41(48)	33(39)
Metastasis	15	06(40)	00(00)	07(47)	07(47)	04(27)	05(33)

+, Positive; T, Tumor size; LN, Lymph node; HG, Histologic grade;
a- $X^2 = 4.46$, r=-0.21, p = 0.03; b- $X^2 = 12.13$, r= -0.35, p=0.002; c-$X^2 = 5.91$, r=+0.17, p=0.05;
d- $X^2 = 15.17$, r=+0.36, p=0.001; e- $X^2 = 7.43$, r=+0.27, p=0.02; f- $X^2 = 7.63$, r=+0.27, p=0.02;
g- $X^2 = 11.55$, r=+0.36, p=0.003

Table 2: Correlation of clinicopathological parameters in non-basal and basal-like triple negative breast cancer.

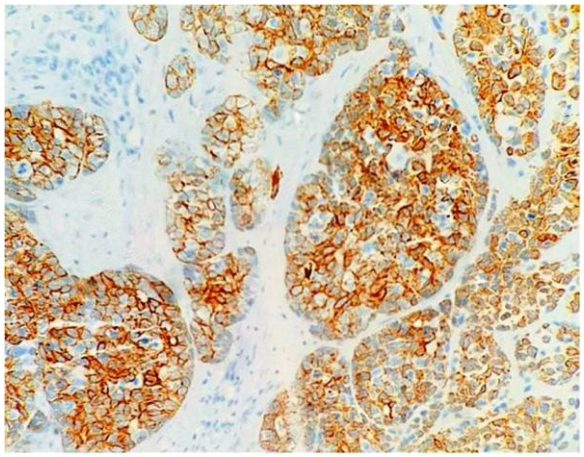
Parameter	N =100	Non-Basal TNBC	Basal Like TNBC
Age <45 years	58	14(24)	44(76)
Age ≥45 years	42	07(17)	35(83)
Premenopausal	35	05(14)	30(86)
Postmenopausal	65	16(25)	49(75)
T1	08	01(12)	07(88)
T2	84	18(21)	66(79)
T3	08	02(25)	06(75)
LN Negative	62	11(18)	51(82)
LN Positive	38	10(26)	28(74)
Stage I	02	00(00)	02(100)
Stage II	87	17(19)	70(81)
Stage III	11	04(36)	07(64)
HG I	07	01(14)	06(86)
HG II	54	16(30)	38(70)
HG III	39	04(10)	35(90)
BR Score (N=85) Low	11	04(36) [a]	07(64)[a]
BR Score Intermediate	45	11(24) [a]	34(76) [a]
BR Score High	29	02(07) [a]	27(93) [a]
No Metastasis	85	17(20)	68(80)
Metastasis	15	04(27)	11(73)

T, Tumor size; LN, Lymph node; HG, Histologic grade;
a- $X^2 = 5.50$, r=+0.25, p = 0.02

Table 3: Kaplan and Meier survival analysis for relapse free survival in relation to marker expression.

Parameter	N	Number of patients relapsed	Log Rank	df	p
CK5/6-	64	09(14)	0.02	1	0.86
CK5/6+	36	06(17)			
CK14-	96	15(16)	0.36	1	0.54
CK14+	04	00(00)			
CK17-	55	08(14)	0.01	1	0.89
CK17+	45	07(16)			
Vimentin-	50	08(16)	0.05	1	0.47
Vimentin+	50	07(14)			
EGFR-	55	11(20)	2.82	1	0.09
EGFR+	45	04(09)			
C-kit-	62	10(16)	0.54	1	0.46
Ckit+	38	05(13)			
Basal markers-	23	04(17)	0.17	1	0.67
Basal markers+	77	11(14)			

-, negative; +, Positive;



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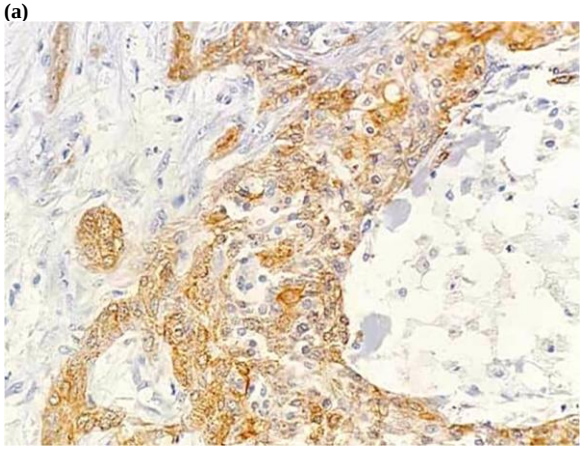

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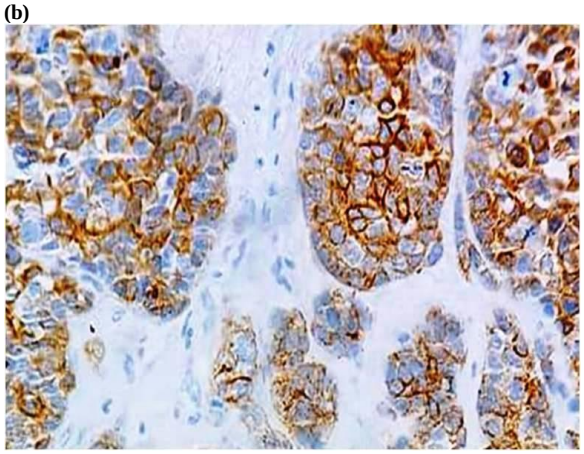

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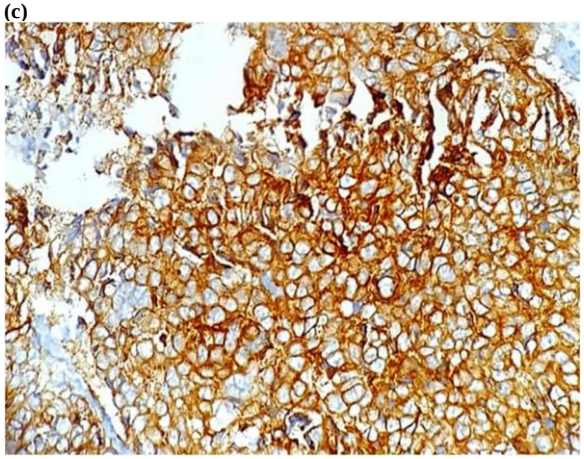

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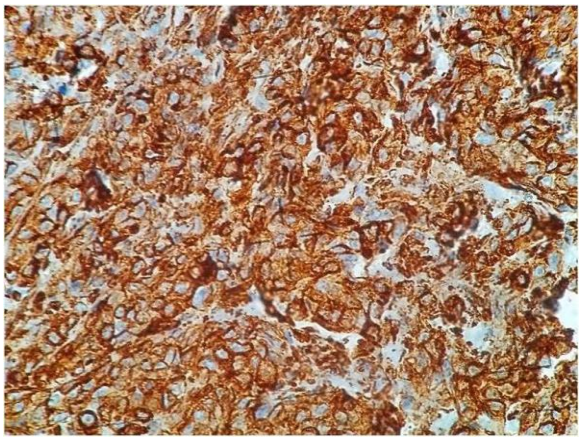
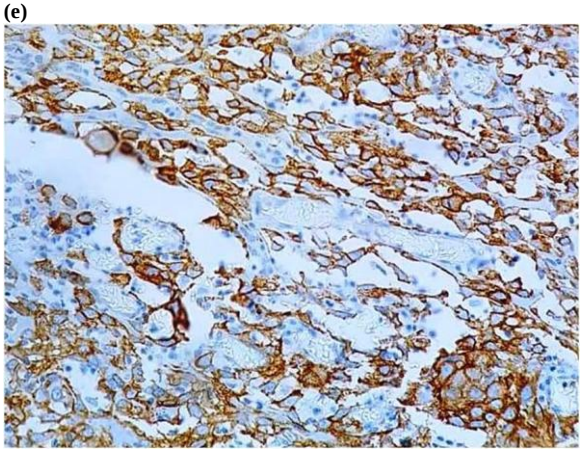
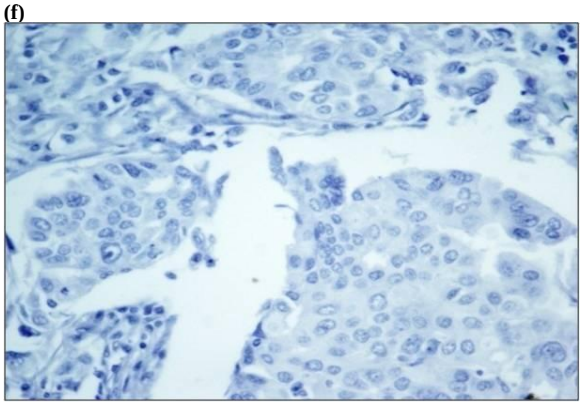


(e)



(f)



(g)

Figure 1: Immunohistochemical staining of CK5/6 (a), CK14 (b), CK17 (c), EGFR (d), Vimentin (e), C-kit (f), and Negative Control (g).

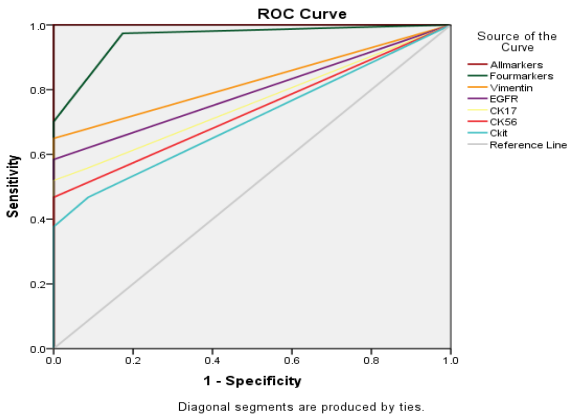


Figure 2: ROC curve analysis of markers

C-kit expression

Cytoplasmic expression of C-kit was noted in tumor tissue of 38% of the patients with an intensity of 1+, 2+ and 3+ in 9%, 14%, and 15% of the patients, respectively (Figure 1f).

In relation to clinicopathological parameters, C-kit expression was found significantly higher in histologic grade III tumors. A similar trend was seen in higher BR score tumors. In relation to histological type, 45% patients with IDC exhibited C-kit expression (Table 1).

Basal vs Non-Basal

All TNBC patients were divided into Basal and Non-basal group upon expression of basal markers expression (CK5/6, Ki67, EGFR, Vimentin and CK 17). Patient positive for even a single marker was considered as basal and that of negative for all was considered as non- basal. Out of 100 TNBC patients, 77% were positive for basal marker expression (Basal like) and 23% were negative for basal marker expression (Non-Basal) (Table 2).

ROC Curve Analysis

ROC curve analysis of individual markers indicated Vimentin (P=0.001), EGFR (P=0.001), CK17 (P=0.001), CK5/6 (P=0.01) and C-Kit (P=0.003), and could be used for identification of Basal-like TNBCs (Figure 6). These four markers combination showed AUC=0.986, 95% CI 0.963 - 1.000, P=0.0001, and all six markers combination showed AUC=1.00, 95% CI 1.00 – 1.00, P=0.0001 yielding a higher statistically significance than individual marker for identification of Basal-like TNBCs.

Further, multivariate analysis by General Linear Model indicated that four markers combination (Vimentin, EGFR, CK17, CK5/6) has maximum 1.00 observed power of the test for identification of Basal-like TNBCs.

Univariate and Multivariate survival analysis

In univariate survival analysis, individual markers and combination of markers did not discriminate patients with better or worse disease free survival (Table 3). In multivariate survival analysis using Cox regression model with forward stepwise (likelihood ratio) method was carried out to evaluate the prognostic significance of clinicopathological parameters and newer markers such as age, menopausal status, tumor size, lymph node status, disease stage, histopathology, histologic grade, BR score, CK5/6, CK14, EGFR, vimentin, CK17, C-kit, Four markers and All markers, disease stage entered at step 1 as significant prognostic factor (Wald=7.12, df=1, p=0.008, Exp (B) 4.63).

Inter-marker Correlation

All the makers included in the study were intercorrelated with each other, a significant positive correlation was noted between CK5/6 with CK14 (r = 0.27, p = 0.006), CK17 (r = 0.76, p = 0.0001), Vimentin (r = 0.20, p = 0.038), and EGFR (r = 0.28, p = 0.004); CK17 with CK14 (r=0.22, P=0.02), EGFR (r = 0.23, p = 0.02), and C-kit (r=0.36, p=0.0001); Vimentin with EGFR (r = 0.23, p = 0.02), and C-kit (r = 0.28, p = 0.004), as well as EGFR with C-kit (r = 0.23, p = 0.004).

DISCUSSION

The present study, evaluated basal markers such as CK5/6, CK14, CK17, Vimentin and EGFR, along with C-kit in TNBC. Regarding individual basal marker expression, CK5/6, CK14, CK17, Vimentin and EGFR expression was seen in 36%, 4%, 45%, 50% and 45% TNBC, respectively. Single basal marker expression was seen in 27%, dual in 23%, triple in 20% and all four markers in 9% patients. As the incidence of CK14 expression was very low (4%), CK5/6, CK17, EGFR and Vimentin were clubbed to identify basal like triple negative breast cancer. Seventy nine percent patients expressed basal markers and identified as basal like triple negative breast cancer

whereas 21% patients did not expressed basal markers and identified as non-basal triple negative breast cancer. These findings are in accordance with data available in triple negative molecular subtype, 70-90% tumors are basal like. [4,5,6] A study by Lesar et al also observed similar distribution like us of basal like (77.3%) and nonbasal like (22.67%) TNBC by immunohistochemical analysis using CK5/6, CK14 and P-cadherin markers [4] and suggested high sensitivity of P-cadherin than CK14 and CK5/6 in classification of basal like and non-basal like triple negative breast cancer. [4,7] Majority of the studies identified basal like TNBCs in the range of 68% to 91%. [4,5,6,8,9]

Majority of the studies evaluated sensitivity, specificity, and positive and negative predictive values of these markers except the study of Sabel et al who compared basal marker expression with clinical and histomorphological parameters. [6] Our study correlated marker expression with clinicopathological parameters. The patient's mean and median age at presentation in our study was 50 years and 21% patients were younger than 40 years. This incidence is in concordance with the publish reports from one Indian study by Sabel et al. [6] In relation to clinicopathologic parameters of the present study, in lymph node negative patients significantly higher incidence of CK-17 expression and a trend of higher EGFR expression were seen. With disease advancement a significant decrease of incidence of CK-17 expression and a trend of decrease in incidence of combination of all four basal markers were seen. Further with advance histologic grade of the tumor and high BR score tumors, increased incidence of Vimentin, EGFR and combination of all markers expression was found. C-kit expression was seen in 38% of patients and an increasing trend of expression was seen with advance histologic grade of the tumor and high BR score tumors.

By ROC curve analysis, combination of four markers Vimentin, EGFR, CK17, and CK5/6, yield higher statistical significance in classifying basal like triple negative breast cancer (P=0.0001) with a maximum power of test 1.00. A study by Sousa et al evaluated sensitivity of markers in pairs and have shown highest sensitivity of P-cadherin/CK5 (97.3%) followed by CK5/Vimentin (94.6%) and CK5/CK14 (91.9%). [7] Regarding disease outcome, in this study majority of patients were of stage II disease (N=87), hence, we did not get statistical significance between marker expression and disease free survival and overall survival.

CONCLUSION

In conclusion, a cost effective panel of immunohistochemistry markers includes Vimentin, EGFR, CK17 and CK5/6 can be used for classification of basal like TNBCs and may be useful in deciding therapeutic strategy.

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