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Original Research Article

Is a Five Marker Panel including Expression of Basal Markers (EGFR and/or CK5/6) compared to Three Marker Panel better in Predicting Prognosis in Molecular Subtypes of Breast Carcinoma?

Trivedi Pawan[1], Varma Kachnar[2], Dhingra Vishal[3], Singh Premala A[4], MisraVatsala[5], Srivastva Sapan*
[1]Junior Resident, Dept of Pathology, MLN Medical College, Allahabad
[2]Associate Professor, Dept of Pathology, MLN Medical College, Allahabad.
[3]Assistant Professor, Dept of Pathology, MLN Medical College, Allahabad
[4]Professor, Dept of Pathology, MLN Medical College, Allahabad
[5]Professor, Dept of Pathology MLN Medical College, Allahabad
*Consultant Oncosurgeon, Kamla Nehru Memorial Hospital, Allahabad

***Corresponding author**
Pawan Trivedi
Email: drpwn1@gmail.com

Abstract: Breast cancer shows marked heterogeneity which is proven by the fact that tumors with similar morphologic and immuno histo-chemical features show distinct clinical behavior and different response to therapy. This led to microarray-based global gene expression profiling (GEP) and new avenues for classifying breast cancer into molecular subtypes. Among all molecular subtypes, the worst prognosis group has been identified as triple negative phenotype (TN). Further within this group, basal like breast cancer (BLBC) was identified using a 5 marker surrogate panel including ER-PR-HER2–negative and basal markers i.e. epidermal growth factor receptor (EGFR) or Cytokeratin 5/6 (CK5/6) positive. EGFR and CK 5/6 are easily available and specific IHC surrogate basal markers and can be readily included in a five marker panel in prognostication of breast cancers. BME is not limited to triple negative subtypes but is also seen in other molecular subtypes. 106 cases of invasive breast carcinoma in which detailed clinical and histological prognostic factors could be determined were classified into molecular phenotype using IHC surrogate classification. Tumors expressing basal markers CK5/6 and EGFR were classified as basal marker expressing (BME) tumors and were also compared with ER, PR, Her-2/neu expressing and also triple negative tumors. These tumors were compared with various prognostic and predictive markers of invasive breast carcinoma. BME was seen in 50/106 cases. Also BME showed a significant association with tumor necrosis, lymph node metastasis and high histological grade. BME in breast carcinomas is an independent prognostic marker and its expression is not limited to triple negative cases. An expanded surrogate panel of ER, PR, Her-2 neu, EGFR and CK 5/6 provides more prognostic value than three panel marker.
Keywords: Breast cancer, basal marker expression, triple negative, EGFR, CK 5/6

INTRODUCTION

Breast cancer is the most common cancer in women worldwide and only second to lung cancer. It is a heterogeneous disease and shows many histological patterns. In the recent years with better understanding of genetic profile it has been seen that tumors with similar histology show different clinical behavior, hence there was a need for classification of breast cancer into subgroups based on the gene expression profile (GEP) came up [1]. Based on the study of these profiles, breast cancer can be divided into five subtypes: luminal A, luminal B, Triple negative (TN) basal-like, normal breast like and human epidermal growth factor receptor 2 (HER2)-over expressing subtype [1]. Among these, basal like subtype, which account for 15 to 20% of all breast cancers are of particular importance as they confer markedly poor prognosis [2].

Luminal-like cancers are Estrogen (ER)/Progesterone(PR)positive with lower grade, and therefore they are sensitive to endocrine therapy and have a more favourable prognosis than the ER-negative and high-grade basal-like cancers(BLBC)[1]. BLBC is a subtype of TN breast cancer identified using a 5 panel biomarker that are negative for ER, PR, HER2 and positive for epidermal growth factor receptor (EGFR)

and/ or Cytokeratin 5/6 (CK5/6). These tumors are associated with high grade, younger age group, poor response to chemotherapy and thereby portend poor prognosis[3]. Role of basal markers has been extensively studied by numerous studies in TN tumors [3-6]. However expression of basal markers is not limited to TN tumors but basal marker expression (BME) is also seen in other molecular subtypes especially Her-2 OE[4]. Better understanding of the role of basal markers in breast carcinoma as prognostic markers and their importance in developing specific therapeutic regimen needs to be explored in non-TN breast subtypes also.

Hence this study was conducted in which a 5-panel IHC surrogate panel was used to classify invasive breast carcinomas into molecular sub-classes as IHC surrogate panels are now available which correspond to the initial gene expression profiling studies[7]. Tumors expressing basal markers were compared with conventional prognostic and predictive markers as well as with recent biomarkers. Statistical analysis was done to find out significant association between the two.

MATERIALS AND METHOD

The present study was conducted in the Department of Pathology, Moti Lal Nehru Medical College, Allahabad, India between August 2013 and August 2015. The test population consists of 106 patients of invasive breast carcinoma who underwent radical mastectomy. Clinical information regarding age, menopausal status, cancer characteristics and nodal disease status was noted. Detailed histological features and other prognostic parameters were noted, a five panel IHC surrogate panel – ER, PR, Her-2/neu and two basal markers CK5/6 and EGFR was done.

IHC based classification corresponding to all molecular classes are being used to define the molecular sub-types of breast cancer, have been documented in various studies as given below.

Reference with direct correlation to expression profiling	**Immunohistochemical classes and criteria**
Nielsen	Basal-like: ER-, HER2 – to low, CK5/6+, and/or EGFR+
Livasy	Basal-like: ER-, HER2 –, CK5/6+, and/or EGFR+ Luminal: ER+, HER2- HER2+: ER-, HER2+
Carey	Luminal A: ER+, and/or PgR+, HER2- Luminal B: ER+ and/or PgR+, HER2+ Basal-like: ER-, PgR-, HER2-, CK5/6+ and/or EGFR+ HER2+: ER-, PgR-, HER2+ Unclassified: negative for all five markers
Cheang	Luminal: ER+ and /or PgR+, and HER2- Luminal+/HER2+: ER+ and/or PgR+, and HER2+ HER2+/ER-, PgR-: HER2+ and negative for ER and PgR Core basal: ER-, PgR-, HER2-, either CK5/6+ or EGFR+ Five negative phenotype: ER-, PgR-, HER2-, also negative for CK5/6 and EGFR
Bhargava	Luminal A: ER+(s), HER2- Luminal B: ER+(w/m), HER2- ERBB2: ER-, PgR-, HER2+ TN: ER-, PgR-, HER2- LAHH: ER+(s), HER2+ LBHH: ER+(w/m), HER2+

LAHH, luminal 1-HER2 hybrid; LBHH, luminal B-HER2 hybrid; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; (s), strong; w/m, weak to moderate.

In above studies, it has been proven that IHC based molecular sub classification do correspond to gene expression profiling studies. These molecular classes are similar although not identical to GEP based molecular classification.

Molecular sub types used in this study	Criteria used for the IHC categories
Luminal A	ER score 200 or higher, HER2 negative
Luminal B	ER score 11-199 or PR score >10, HER2 negative
ERBB2/HER2 OE	ER and PR score 10 or less, HER2 positive
Triple negative	ER and PR score 10 or less, HER2 negative
Basal like	CK 5/6 and/or EGFR positive

We used IHC surrogate criteria proposed by Bhargava *et al.;* [7] to sub-classify breast cancer into molecular subtypes. We could not do GEP studies as our center does not have this facility and also we did not have any funding for this study.

Estrogen receptor (ER) and progesterone receptor (PR) results were reported using a semi-quantitative score (previously described as ''H-score'') [8], which details the percentage of positive cells showing none, weak, moderate, or strong staining. The score is given as the sum of the percentage staining multiplied by an ordinal value corresponding to the intensity level (0 = none, 1 = weak, 2 = moderate, 3 = strong). With 4 intensity levels, the resulting score ranges from 0 (no staining in the tumor) to 300 (diffuse intense staining of the tumor).

For positive control we used normal breast tissue and for negative control we performed IHC without applying primary antibody with each lot of IHC staining. All cases in this study were mastectomy specimen and for IHC, sections having tumour area as well as adjacent normal breast, were used.

Antibody	Clone	Dilution	Company
ER	ID5	Prediluted	BioGenex
PR	PR88	Prediluted	BioGenex

For Her2-neu, FDA Scoring Criteria was used. EGFR and CK5/6 stains were considered positive if any (weak estrogen) cytoplasmic and/or membranous invasive carcinoma cell staining was observed. Using IHC surrogates, five molecular subtypes were defined. (Table 2)

Statistical analysis was done by applying chi square test and calculated the *P value* using SPSS software.

RESULTS

The present study comprises of 106 cases of invasive carcinoma breast. As shown in Table 2, out of 106 invasive breast carcinomas, 51.9% cases (55/106)showed BME. 29 (80.5%) TN sub type followed by Her-2neu (22/32; 68.7%), followed by luminal B (22%) and luminal A (4%) were also BME.

BME expressing tumors were compared with conventional prognostic markers i.e. age, tumor necrosis, lymph node (LN) metastasis and tumor grade (Table 3). As seen in Table, a statically significant association was found between younger age group, tumor necrosis, LN metastasis and higher grade. 65.4% cases in ≤50 years' age group were basal positive. 67.3% cases associated with tumor necrosis were showing BME. As far as LN metastasis is concerned, 69.2% cases of positive LN disease were BME. Most of the high MBR grade (Grade 2 and 3) tumors were expressing basal markers. Also BME expressing tumors were compared with ER, PR status, Her-2 OE and TN phenotype as shown in Table 4. A Statically significant association was found between BME tumors and Her-2 neu OE and TN tumors (TN). Among the ER and PR expressing tumors, BME was higher in PR positive tumors (44.7%) in comparison to ER positive tumors (40.5%).

Table 1: Showing IHC clones used in present study

Antibody	Clone	Dilution	Company
ER	ID5	Prediluted	BioGenex
PR	PR88	Prediluted	BioGenex
HER-2	EP1045Y	Prediluted	BioGenex
EGFR	Polyclonal	Prediluted	BioGenex
CK5/6	EPR1600Y & EPR1602Y	Prediluted	BioGenex

Table 2: Showing distribution of cases into molecular subclasses using a panel of 5 IHC markers

Molecular class	N=106	EGFR	CK 5/6	Cases showing BME N=50
Luminal A	24 (22.6%)	1	1	1 (4.1%)
Luminal B	14 (13.2%)	2	3	3 (21.7%)
Her 2 OE	32 (30.2%)	18	16	20 (62.5%)
TN	36 (33%)	12	22	26 (71.4%)

Table 3: Showing distribution of cases according to basal marker expressing (BME) and non-basal marker expressing tumors and various prognostic markers

n	Age (yrs.)			Tumor size(cm)			Necrosis			LN Mets.			Grade			
	≤50	>50	*P*	≤2	>2	*P*	+	-	*P*	+	-	*P*	I	II	III	*P*
BME (55)	38 (69.1)	17 (30.9)	**0.000045**	27 (49.1)	28 (50.9)	0.845	37 (67.3)	18 (32.7)	**0.000098**	36 (65.4)	19 (34.6)	**0.000453**	3 (5.4)	25 (45.4)	27 (49.2)	**0.000137**
Non-BME (51)	15 (29.4)	36 (70.6)		26 (51)	25 (49)		15 (29.4)	36 (70.6)		16 (31.4)	35 (68.6)		16 (31.4)	26 (51)	9 (17.6)	

Table 4: Showing relation between Basal Marker Expressing tumors and ER, PR, Her-2 neu and TN status.

N	ER			PR			Her-2/neu			TN		
	+	-	*P*	+	-	*P*	+	-	*P*	+	-	*P*
BME (50)	15	40	0.0868	17	38	0.270	24	31	**0.0017**	26	29	**0.0026**
Non-BME (56)	22	29		21	30		08	43		10	41	

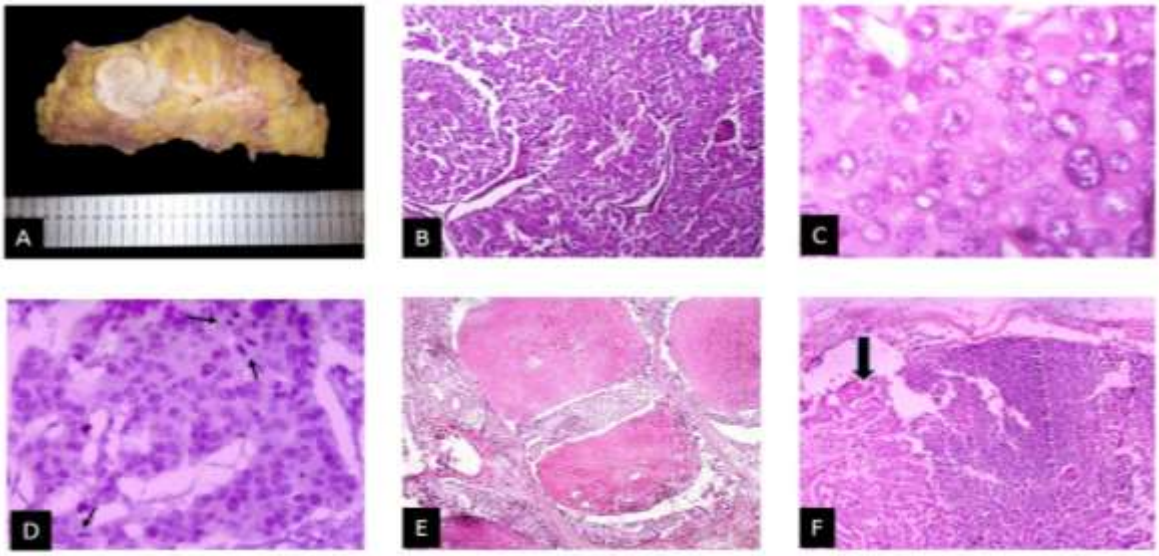

Fig 1: Photomicrographs of Invasive Ductal Carcinoma - No Special Type with Basal Marker Expression.
(A) Gross specimen showing cut surface of grey-white tumour mass with infiltrative borders,
(B) Tubule formation < 10%, mainly tumor cells in diffuse sheets. (H and E ×40),
(C) Marked nuclear pleomorphism (H and E ×400),
(D) Frequent mitotic figures (arrow) (H and E ×100),
(E) Comedo pattern of extensive tumour necrosis (H and E ×40),
(F) Metastasis to Axillary lymph node. (arrow) (H and E×40).

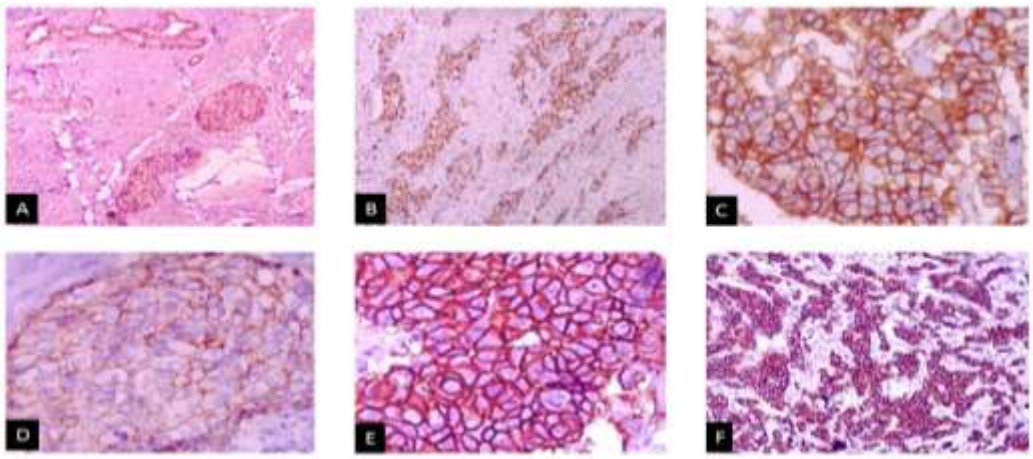

Fig 2: Photomicrographs showing
(A) Strong nuclear positivity for ER by tumour cells (IHC ×100),
(B) Strong nuclear positivity for PR by tumour cells (IHC ×100),
(C) Strong and complete membrane positivity for Her-2 (IHC ×400),
(D) Weak and incomplete membrane positivity for Her-2 (IHC ×400),
(E) Strong and complete membrane positivity for EGFR (IHC ×400),
(F) Strong and complete membranous positivity for CK 5/6 (IHC ×100).

DISCUSSION

As breast cancer shows remarkable heterogeneity therefore the need arose for recent classification of breast cancer into subgroups based on the gene expression profile [1]. Based on the study of these profiles, breast cancer can be divided into five subtypes: luminal A, luminal B, TN basal-like, normal-likeandHER2-OE subtype. Of particular importance is the BLBC, which accounts for 15 to 20% of all breast cancers and confers a markedly poor prognosis. BLBC was identified using a 5 panel biomarker including ER-PR-HER2–negative and epidermal growth factor receptor (EGFR) or Cytokeratin 5/6 (CK5/6) positive. This category has proved to be of much clinical importance as these tumors are associated with high grade, younger age group and poor response to chemotherapy [3-8].

In normal breast tissue, the term basal has been applied to the well-defined myoepithelial (contractile) cells and basal CK-expressing cells that may be found in either a luminal or basal location [4, 9]. At the DNA level, basal-like tumors show the most frequent chromosomal gains and losses, less-frequent DNA amplification and a higher rate of loss of heterozygosity than other subtypes. These tumors seem to Harbor early onset (BRCA1) pathway [10, 11]. CK5/6 and EGFR are specific basal markers with prognostic implications. CK 5/6 expression in breast carcinoma implies a 'basal like' molecular phenotype and is associated with poor prognosis [12].

EGFR is a 170-kDa membrane-bound tyrosine kinase. The EGFR protein product has an important role in cell proliferation, migration, and protection against apoptosis mediated by subsequent activation of intracellular pathways [13]. The poorer prognosis of breast carcinomas expressing EGFR is likely connected to these functions. Targeted anti-EGFR antibodies (eg, cetuximab) and EGFR tyrosine kinase inhibitors (eg, gefitinib) may provide a possible treatment modality. An association of a high EGFR intratumoral level with shorter survival was seen not only in TN breast carcinoma but also in non-TN breast carcinomas [14, 15].

In our study, BME was seen in both TN and Non TN cases as seen in Table 2. BME was seen in 29 (80.5%) TN sub type followed by Her-2neu (22/32; 68.7%), followed by luminal B (22%) and luminal A (4%). BME was maximum in TN category followed by Her-2 OE tumors both of which classes show worse prognosis. A subgroup of HER2-OE tumors that show BME-the so-called basal-HER2+ subtype--is associated with poor prognosis [16]. This subtype highlights the heterogeneous biology of this group and is independently associated with poor survival and may provide insight into breast cancer cell response to anti-HER2 therapy [17].

Luminal subtype A and B breast cancer cells are ER^+ and/or PR^+ and patients with these two types of breast cancer are treated with endocrine therapy such as tamoxifen, to inhibit the function of ER [18]. However, in ER^+ patients, endocrine therapy is effective in only 30% of cases as different signaling pathways may be activated [19]. Thus for ER^+ breast cancers, different molecular subtypes have been further described, such as the five-biomarker panel signature by ER, PR, HER2, CK 5/6 and EGFR. The primary considerations

regarding treatment options in these cases may be the EGFR, or IGF-1, VEGF and PI3K/AKT signaling pathway components. Knowledge of these pathways in tamoxifen resistant cases can lead to other therapeutic strategies, such as treatment with the anti-VEGF antibody bevacizumab combined with paclitaxel [20].

The above studies predicting poor prognosis of BME tumors further correlated with our study. We also found that these tumors correlated significantly with younger age of the patients, presence of tumor necrosis, LN positive disease and high histological grade. Earlier studies have shown significant association of tumor necrosis, axillary lymph node positivity, high tumor grade in BLBC tumors but importance of BME in Non-TN tumors lies unexplored [5, 6, 21].

Most of the cases in this study were lost to follow up as they have referred to higher/oncology centre for further treatment, so status of metastasis was not known and we could not correlate BME with TNM staging. However, correlation of BME with tumour size and axillary lymph node was not significant.

Out of 106 only 21 cases could be followed up for 1 year and rest were lost to follow up. Out of these 21 cases one case had local recurrence. This case was basal marker positive.

CONCLUSION

Although presently, role of basal markers has been explored only in TN breast cancers, in future they may have a role as a predictor of worse prognosis in non-TN tumors also. It may inform the clinician of tumors likely failure to respond to hormoneor HER2-targeted therapy.

Moreover, other tailored therapy options may be available for patients with BME cancers, such as the tyrosine kinase inhibitors, anti-EGFR or anti-SRC, and TRAIL inhibitors [22, 23]. Thus a routinely available 5 panel which could be easily done on formalin-fixed, paraffin blocks, could identify a separate cohort of breast cancer patients expressing basal markers.

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