Clinico-Pathological Characteristics of Triple Negative and Non Triple Negative High Grade Breast Carcinomas with and Without Basal Marker (CK5/6 and EGFR) Expression at a Rural Tertiary Hospital in India

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Abstract: Aims of the study were to evaluate the expression Cytokeratin 5/6(CK5/6) and Epidermal Growth Factor Receptor (EGFR) among triple negative breast cancers and high grade infiltrating duct carcinomas. Further to probe if triple negative phenotype can be a surrogate marker for basal phenotype and to correlate the expression of basal markers with disease free survivals among triple negative phenotype and high grade infiltrating duct carcinomas.

Methods: Expression of CK5/6 and EGFR were studied by Immunohistochemistry (IHC) in 31 triple negative and 19 non-triple negative high grade breast carcinomas.

Results: 21 of the 31 triple negative phenotype (67.7%) breast carcinomas and 7 out of 19 non-triple negative (36.8%) breast carcinomas showed expression of basal markers (CK5/6 and/or over-expression of EGFR). There were statistically significant associations of all the basal-like tumors with negative hormonal status. The basal markers positive phenotype subjects had a shorter disease free interval as compared to basal markers negative phenotype subjects.

Conclusion: Basal-like breast carcinomas constitute a unique clinical and pathological entity, characterized by high tumor grade and a propensity for lack of ER, PR and HER2 expression. Basal phenotypes have a more aggressive course than non-basal phenotype. “Triple negative” status cannot be used as a surrogate for “basal marker expression”.

Keywords: breast cancer, tumor grade, basal-like, triple-negative, CK5/6, EGFR
Introduction

Breast cancer is a multifaceted disease comprising of distinct biological subtypes with diverse natural history, presenting a varied spectrum of clinical, pathologic and molecular features with different prognostic and therapeutic implications. Recent attention has been directed singularly at molecular classifications of breast cancer.1,2

Currently, routine clinical management of breast cancer incorporates specific molecular markers; namely ER-estrogen receptor, PR-progesterone receptor, HER2-human epidermal growth factor receptor 2 gene, that have been proven to provide therapeutic, predictive and prognostic value.3

Global gene expression profiles (GEP) has led to the identification of five molecular breast cancer subtypes (Table 1), of which only four have been well defined while the fifth being “normal mammary stromal cells like” is poorly defined.

The hormonal positive group (ER and/or PR positive) of tumors are clustered into a large group named ‘luminal class’ which is further sub-divided into Luminal A and Luminal B depending on presence or absence of HER2 positivity. The hormonal negative group of tumors is comprised of

1. HER-2+ type: Tumors with gene characteristics of HER2 amplification
2. Basal type: Tumors with gene expression that is similar to normal basal/myoepithelial cells
3. Normal breast stromal cells like tumors that lack hormonal group, HER2 amplification and basal-like expression profile which are similar to normal mammary stromal cells.3,4

Breast cancers ER/PR−, HER2− phenotype are classified as triple negative/basal-like tumors3 and have poor prognosis and therapy response.5

Although an ER negative/PR negative/HER-2 negative (ER-/PR-/HER-2-) or “triple negative” immunophenotype is considered sufficient to identify basal-like tumors, increasing evidence shows that “basal-like” and “triple negative” are not synonymous. A more complete definition should include expression of basal cytokeratins (CK5/6, 14, 17) and/or over-expression of human epidermal growth factor receptor 1 (HER-1).6 This is further supported by recent evidence suggesting that triple negative breast carcinomas represent a more heterogeneous group than basal-like tumors.7 The aggressive behavior of these triple negative breast tumors is due to the co-expression of basal markers.8

Basal cytokeratins (CK) represent a large number of high molecular weight (HMW) cytokeratins mainly seen in the basal cell layers of stratified epithelium.9 In the human breast, these cytokeratins are also expressed in the basally-located myo-epithelial cell layer and in a small proportion of luminal epithelial cells of the glands.10 Breast tumors characterized by CK5, CK14 and CK17 expression are classified as basal phenotype. As these markers were seen in the basal layers, “basalness” was often interpreted as a sign of a myo-epithelial origin which created confusion. However it has been observed that not only grade 3 invasive ductal carcinomas (NOS), but also other histological breast cancer subgroups, such as metaplastic and medullary carcinomas, with or without associated BRCA1 mutations also expressed CK5/6, a basal marker.11 In contrast to all other breast cancer subgroups, metaplastic subgroups harbor EGFR-amplifications,11 more commonly than other types of breast cancer.12 Thus, breast carcinomas have been deemed basal when they express HMW-cytokeratin even in a single malignant cell.12 “Basal phenotypes” of breast carcinomas are usually ER, PR

Table 1. Types of invasive breast carcinomas based on gene expression profiling (GEP).

| Hormonal class        | Molecular sub-type              | ER and PR expression | HER2-neu expression | Basal marker expression |
|-----------------------|--------------------------------|----------------------|---------------------|------------------------|
| Hormone positive      | Luminal A                       | ER/PR +ve            | HER-2 negative      | Basal markers +ve      |
| “luminal class”       | Luminal B                       | ER/PR +ve            | HER-2 positive      | Basal markers +ve      |
| Hormone negative      | HER-2                           | ER/PR −ve            | HER-2 negative      | Basal markers −ve      |
| “nonluminal class”    | Triple negative                  | ER/PR −ve            | HER-2 negative      | Basal markers −ve      |
|                       | Normal breast stromal cells like | ER/PR −ve            |                     |                        |
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and HER-2 neu negative and have been consistently associated with expression of epidermal growth factor receptor (EGFR), c-kit, p53, and p63. Basal breast cancer requires a set of diagnostic markers, and has been defined differently in different studies. In 2009, Rakha and Ellis recommended 4 basal markers, namely C5/6, CK14, CK17 and EGFR of which at least 2 should be positive to be termed as Basal-like breast cancer.

The present study has been undertaken to:

1. Evaluate the expression of two basal markers (CK5/6 and EGFR) among triple negative breast cancers and high grade infiltrating duct carcinomas.
2. Study whether triple negative phenotype (IHC profile) can be a surrogate marker (profile) for basal phenotype.
3. Correlate the expression of basal markers with disease free survivals among triple negative phenotype and high grade infiltrating duct carcinomas.

Materials and Methods

A total of 50 cases of either triple negative or high grade (primary and recurrent) invasive breast carcinoma were retrieved from the files of Pathology Department, Kasturba Medical College Hospital, Manipal University, Manipal, India. Patients were diagnosed and treated in our institution between January 1, 2006 and December 31, 2007. Information regarding age, menopausal status, cancer characteristics, stage of cancer at diagnosis, nodal disease status, periductal elastosis, specifics of treatment, recurrence, date and location of recurrence, date and cause of death, and length of survival were obtained from case records. The medical records of the subjects included in the study were followed up until August 1, 2009. This study was approved by the Institutional Ethics Committee.

Resources

Pathological diagnosis

All surgical tissue specimens were fixed in 10% formaldehyde, embedded in paraffin, sectioned and stained with Hematoxylin/Eosin. The tumors were classified and graded according to World Health Organization classification and the Nottingham modification of the (Scarff-Bloom-Richardson) SBR system, respectively.

Immuno-histochemical staining

Four micrometer sections attached on poly-lysine coated slides and incubated overnight at 37 °C. The slides were dewaxed in xylene, rehydrated in graded ethanol, distilled water and covered with 10 mM citrate buffer (pH 6). They were then incubated for 30 minutes with primary monoclonal anti-bodies against HER2 (DAKO, clone 250, 1:100, antigen retrieval: 2 min pressure cooker), ER (DAKO, clone SP1, 1:50, antigen retrieval: 2 minutes pressure cooker) and PR (DAKO, clone PgR636, 1:50, antigen retrieval: 2 minutes pressure cooker), CK5/CK6 (DAKO, clone D5/16B4, 1:50, antigen retrieval: 18 min microwave oven), EGFR(DAKO clone 2-18c9, 1:40, antigen retrieval: proteinase K) followed by incubation with biotin-labeled secondary antibodies. The streptavidin-peroxidase complex was visualized using di-aminobenzidine as a chromogenic substrate. For each run of staining, a positive control slide was prepared from breast carcinoma known to be positive for the proteins studied.

ER/PR

The sections were assessed for ER and PR by Quick score. For the current study 1% of the cells showing ER positivity were considered as positive.

HER2-Neu

The sections were assessed for HER2 membrane staining according to American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer.

For the current study, complete, dark membrane staining in >30% of the cells was scored as 3+ and was taken as positive. Less than 30% of the cells with complete dark membrane positivity/or incomplete light membrane staining was scored as 2+ and was considered negative. However they were advised to look for over-amplification of HER2 neu by fluorescent in-situ hybridization (FISH).

Case Selection

Based on the immunohistochemical staining status (ER, PR and HER2), we included 31 triple negative phenotype [ER/PR-, HER2-] breast carcinomas and 19 non-triple negative high grade breast carcinomas (4 Luminal B phenotype [ER/PR+, HER2+]; 13 Luminal A pheno-
type [ER/PR+, HER2−]; 2 HER2 positive phenotype [ER/PR−, HER2+]). Subsequently all the triple negative breast carcinomas and high grade (primary and recurrent) breast carcinomas were assessed for expression of CK5/6 and EGFR.

**EGFR**

Only membrane staining was assessed for EGFR according to DAKO criteria (Table 2).

For the current study, EGFR over-expression of any intensity in more than 1% of cells was considered as positive basal marker. Examples of different staining results are shown in Figure 1A and B.

**CK5/6**

Any cytoplasmic expression in definite neoplastic cells or tissue was considered as positive result. Examples of different staining results are shown in Figure 1C and D.

Pathology slides were reviewed concurrently by two pathologists for grading and evaluating the immunohistochemical stains.

**Statistical Analysis**

The SPSS version 16.0 statistical program was used for analysis. Patient characteristics were compared using Fisher’s exact test. Two tailed P values ≤0.05 were considered significant. Borderline statistical significance was defined as P values between 0.05 and 0.10. Disease free interval was calculated from the date of starting treatment until the recurrence of breast cancer. Survival plots and cumulative survival probabilities were estimated using the Kaplan-Meier method.

**Results**

Twelve out of the thirty one triple negative (basal-like) phenotype [38.7%] breast carcinomas showed CK5/6 expression. Expression of CK5/6 was found only in one non triple negative tumor (Luminal A phenotype).

EGFR over-expression was found in eighteen out of thirty one triple negative (basal-like) phenotype [58.1%] breast carcinomas. Over-expression of EGFR was also found in non triple negative tumors namely 25% of Luminal B phenotype [1 out of 4], 23.1% of Luminal A phenotype [3 out of 13] and 100% of HER2 phenotype [2 out of 2] tumors.

Taken together, twenty one out of the thirty one triple negative (basal-like) phenotype [67.7%] breast carcinomas showed expression of CK5/6 and/or over-expression of EGFR. (Table 3)

There were statistically significant associations of all the basal-like tumors (CK5/6, EGFR, CK5/6 and/or EGFR) with negative hormonal status, and negative estrogen receptor expression. Negative progesterone receptor status was strongly associated only with CK5/6 and basal marker (CK5/6 and/or EGFR) expression. (Table 4)

There was a significant association between negative peri-ductal elastosis and over-expression of EGFR. There was a borderline association between EGFR over-expression and breast carcinomas which showed metaplasia less than 20% of tumor area and all the metaplastic breast tumors. (Table 5)

**Basal Marker Expression and Patient Survival**

Kaplan Meier plots with reference to disease free survivals were similar for triple negative breast cancers expressing EGFR and breast cancers expressing EGFR and/or CK5/6 basal markers (Figure 2A and C). Though not statistically significant, the basal markers positive phenotype subjects had a shorter disease free interval as compared to basal marker negative phenotype. Despite the fact that basal phenotype had shorter disease free interval in the initial follow up period, relatively better disease free interval was noted in the later follow up period, which is similar to the observations made by Arnes et al.15

Disease free survivals for triple negatives expressing C5/6 was different (Figure 1B) from the other two basal marker phenotype. They showed longer disease free interval in the initial period and became shorter in the later part of the follow up.

| Score to report | EGFR assessment | Staining pattern                        |
|----------------|-----------------|-----------------------------------------|
| 1+             | Weak            | Faint and incomplete membrane positivity|
| 2+             | Moderate        | Moderate and complete/incomplete membrane positivity |
| 3+             | Strong          | Strong and complete membrane positivity |

Table 2. EGFR assessment.
Discussion

We examined expression of EGFR and CK5/6 by immunohistochemistry in all the triple negative and high grade breast carcinomas. In the majority of the triple negative breast tumors (21/31, 67.7%), we found expression of at least one of the markers EGFR and/or CK5/6. The individual basal markers namely CK5/6 was expressed in more than a quarter of triple negative tumors (12/31, 38.7%) and EGFR was over-expressed in more than half of the triple negative breast tumors (18/31, 58.1%). Our observations imply a large overlap between “triple negativity” and “basalness”. Similar opinions were expressed by Rakha et al. Our data also shows that EGFR and CK5/6 are also expressed in other immunohistochemical subtypes of breast cancer. Further, CK5/6 was found only in one non triple negative tumor namely Luminal A phenotype breast cancer whereas EGFR was over-expressed in all the HER2 phenotype breast tumors (100%). Recently the “so called basoluminal phenotype” has been described and is characterized by expression of basal markers and HER-2 expression in the luminal cells. In the present study 2 cases of basoluminal phenotype have been documented. Over-expression of EGFR was also seen in a quarter of Luminal B phenotype breast tumors (1/4, 25%) and a little less than a quarter of luminal A phenotype breast tumors (3/13, 23.1%). Though our study population was small, these observations suggest that.

Table 3. Summary of CK5/6 and EGFR expression data by immunohistochemistry.

| Tumour phenotype                  | Basal markers | CK5/6     | EGFR     |
|----------------------------------|---------------|-----------|----------|
| Luminal B                        | 1/4 (25.0)    | 0/4       | 1/4 (25.0) |
| Luminal A                        | 4/13 (30.8)   | 1/13 (7.7)| 3/13 (23.1) |
| HER2                             | 2/2 (100)     | 0/2       | 2/2 (100)  |
| Triple negative (basal like)     | 21/31 (67.7)  | 12/31 (38.7)| 18/31 (58.1) |
| All                              | 28/50 (56.0)  | 13/50 (26.0)| 24/50 (48.0) |

Note: Percentages are given in parentheses.
Abbreviations: CK, cytokeratin; EGFR, epidermal growth factor receptor.
expression of CK5/6 and especially EGFR are not only limited to triple negative phenotype of breast cancers but are also expressed in 22.6% of non-triple negative phenotype of breast cancers (7/19 ie, 25% of luminal B, 30.8% of luminal A, 100% of HER2). In view of the highly complex pathogenesis of breast cancer and presence of basal markers among triple negative and non triple negative breast cancers, it would be imprudent to equate triple negative phenotype to basal-like breast cancers. Larger studies are needed to explore these observations in detail and to study overlap between the different basal markers in different immune-phenotypic subtypes of breast cancer.

In fact, triple negative phenotypic breast cancers are a clinically and molecularly heterogeneous disease that encompasses more than one entity. In addition to basal-like breast tumors, triple negative phenotype tumors also include ‘normal breast-like’ cancers (32.3% of the triple negative phenotype). Similar to our study, a separate basal marker negative, triple negative breast cancer subtype was reported in various studies and was found to have a prognosis which seems to better than basal-like breast cancer.3

Either EGFR over-expression or CK5/6 expression has been included in the definition of a “core basal profile”15. In the present study, the basal marker expression was compared with different prognostic factors. Similar to our observations, there was a borderline association between EGFR over-expression and breast carcinomas which showed metaplastic changes and metaplastic breast carcinomas.11 Breast tumors with metaplastic changes seemed to over express EGFR, however a larger sample size would have produced a significant \( P \) value. A breast tumor was categorized as metaplastic breast carcinoma if metaplastic changes occupied more than 20% of the tumor area11 and categorized as breast carcinoma with metaplastic changes if the metaplastic changes occupied less than 20% of the tumor area. Besides classical sarcomatoid metaplasia, other metaplasias like apocrine, squamous and chondroid were also classified as metaplastic changes in the present study. In concordance with other studies4 all the basal markers showed a significant association with negative estrogen receptor expression and hormonal negative sub-group of breast cancers.

In the present study there was no significant association between basal phenotype and lymph node status, histologic grade, menopausal status, age group and HER2 status. However, our limited study population must be kept in mind before drawing any further conclusion. In the present study, periductal-elastosis was found to be a good predictor of EGFR expression, unlike the conclusion drawn by Remmele et al17 in their study.

The disease-free interval of basal marker positive carcinoma was shorter than that of patients with basal markers negative tumors. Similar observations were made in other studies.3,18 However, CK5/6 positive
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Table 5. Associations between CK5/6, EGFR and basal markers (CK5/6 and/or EGFR) and prognostic factors.

| Prognostic factors       | CK5/6 |       |       | EGFR |       |       | Basal markers |       |       |
|--------------------------|-------|-------|-------|------|-------|-------|---------------|-------|-------|
|                          | +     | –     |       | +    | –     |       |               | +     | –     |
|                          |       |       |       |       |       |       |               |       |       |
|                          |       |       |       |       |       |       |               |       |       |
| Age                      |       |       |       |       |       |       |               |       |       |
| ≥50 years                | 5     | 17    | NS    | 11   | 11    | NS    | 13            | 9     | NS    |
| <50 years                | 8     | 20    |       | 13   | 15    |       | 15            | 13    |       |
| Menopausal status        |       |       |       |       |       |       |               |       |       |
| Peri-menopausal          | 8     | 17    | NS    | 12   | 13    | NS    | 14            | 11    | NS    |
| Post-menopausal          | 5     | 20    |       | 12   | 13    |       | 14            | 11    |       |
| Histologic type          |       |       |       |       |       |       |               |       |       |
| IDC-nos                  | 12    | 36    | NS    | 23   | 25    | NS    | 26            | 22    | NS    |
| IDC-special type         | 1     | 1     |       | 1    | 1     |       | 2             | 0     |       |
| Histologic grade         |       |       |       |       |       |       |               |       |       |
| Grade 1 and 2            | 2     | 4     | NS    | 4    | 2     | NS    | 5             | 1     | NS    |
| Grade 3                  | 21    | 23    |       | 20   | 24    |       | 23            | 21    |       |
| Periductal elastosis     |       |       |       |       |       |       |               |       |       |
| Present                  | 0     | 7     | NS    | 1    | 6     | 0.037 | 1             | 6     | 0.014 |
| Negative                 | 12    | 21    |       | 20   | 13    |       | 23            | 10    |       |
| Lymph node status        |       |       |       |       |       |       |               |       |       |
| Positive                 | 6     | 23    | NS    | 14   | 15    | NS    | 16            | 13    | NS    |
| Negative                 | 7     | 14    |       | 10   | 11    |       | 12            | 9     |       |
| Number of positive lymph |       |       |       |       |       |       |               |       |       |
| nodes 1–4                | 4     | 14    | NS    | 8    | 10    | NS    | 10            | 8     | NS    |
| ≥5                       | 2     | 9     |       | 6    | 5     |       | 6             | 5     |       |
| Metaplasia               |       |       |       |       |       |       |               |       |       |
| Present                  | 1     | 7     | NS    | 6    | 2     | 0.095 | 6             | 2     | NS    |
| Absent                   | 12    | 30    |       | 18   | 24    |       | 22            | 20    |       |

Figure 2. Basal marker expression and disease survival. (A) Basal markers (EGFR and/or CK5/6) expression and disease free survival; (B) CK5/6 expression and disease free survival; (C) EGFR expression and disease free survival.
phenotype subjects had a better prognosis compared to basal marker (CK5/6 and/or EGFR) positive phenotype subjects. The number of cases in each sub-group restricts us from drawing definite conclusions. The poor prognosis of basal-like breast carcinomas is noted in the initial days of follow up and then the difference in outcome between basal and non-basal tumors became less evident.

**Conclusion**

Basal-like breast carcinomas constitute a unique clinical and pathological entity, characterized by high tumor grade and a propensity for lack of ER, PR and HER2 expression. However, basal-like carcinomas are not synonymous with triple negative carcinomas. Metaplastic carcinomas are more likely to show basal phenotype. Basal phenotypes have a more aggressive course than non-basal phenotype, as shown by the shorter disease free intervals.

**Disclosures**

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