RESEARCH COMMUNICATION

Immunohistochemical Analysis of ER, PR, Her2 and CK5/6 in Infiltrative Breast Carcinomas in Indian Patients

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Abstract

Aim: Breast cancer is biologically a heterogeneous disease. Patients with the same diagnostic profile have markedly different clinical outcomes. Gene expression studies identified distinct breast cancer subtypes that differ in prognosis. Aim is to identify the immunohistochemical subtypes of breast carcinoma and correlate the results with pathological features associated with adverse prognosis in our study population. Method: We included 107 consecutive cases of invasive breast carcinoma and sub classified using immunohistochemical staining for ER, PR, Her2, and CK5/6 into the following subtypes: luminal A, luminal B, basal-like, Her2$^+$ and unclassified. Associations between tumor subtypes and tumor characteristics were examined. Results: The proportion of each subtype in our patient population was: luminal A 37.4%, luminal B 11.1%, Her2$^+$ 29% and basal-like 7.5%. The following variables were significantly associated with IHC breast cancer subtypes: patient age ($p<.05$), overall histopathology grade ($p<0.001$), nuclear grade ($p<0.005$) and mitotic index ($p<0.001$). Her2$^+$ and basal like subtypes were associated with poor differentiation ($p<0.01$), higher nuclear grade ($p<0.05$) and high mitotic index ($p<0.05$). Conclusions: Our data show a higher proportion of patients in the study population undergo total mastectomy and harbor poorly differentiated, node positive tumors than reported. There was also a relatively high percentage of the Her2$^+$ subtype (29%).

Key Words: Invasive breast carcinoma - immunohistochemistry - molecular subtypes - ER & PR, Her2

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Introduction

Breast cancer is the second most common cancer among women in India, following cancer of the uterine cervix. Presently, 75,000 new cases are reported annually and account for 19-34% of all cancer cases among women nationally (ICMR 1989; Badwe et al., 1990; Murthy et al., 1990; ICMR 2001; Siddqi et al., 2001; Saxena et al., 2005). In the central region of India where this study was undertaken, 50 to 70% of breast cancer patients present in an advanced stage (ICMR 1989).

Breast cancer is a biologically heterogeneous disease and patients with the same diagnostic and clinical prognostic profiles can have markedly different clinical outcomes. This difference is possibly caused by the limitation of our current taxonomy of breast cancers, which groups molecularly distinct diseases into clinical classes based mainly on morphology. A partial explanation for this disparity in behavior is found in studies using modern techniques including molecular profiling to examine the biologic underpinnings of breast cancer (Ahr et al., 2002; Huang et al., 2003).

Molecular profiling has provided biological evidence for heterogeneity of breast cancer through the identification of intrinsic subtypes. Analysis of gene expression data suggest that breast cancers can be divided into molecular subtypes which have distinct clinical features, with markedly differing prognosis and clinical outcomes (Perou et al.,2000; Sorlie et al.,2001; Sorlie et al., 2003; Sotiriou et al.,2003; Nielsen et al., 2004). These subtypes consist of two estrogen receptor (ER) positive types (Luminal A and Luminal B), and three ER-negative types (human epidermal growth factor receptor-2[HER2] expressing, basal like and normal breast-like) (Perou et al., 2000; Sorlie et al., 2001). Luminal A tumors, characterized by positive ER, and negative Her2 show the most favorable clinical features among the five subtypes (Nielson et al., 2004; Carey et al., 2006). Basal-like tumors typically show low expression of ER and HER2, and exhibit high expression of genes characteristic of the basal epithelial cell layer, including expression of cytokeratin 5/6 (CK5/6). This subtype is more prevalent in patients with BRCA1 mutation (Sorlie et al., 2001). The HER2$^+$ tumors fall into at least 2 distinct groups: HER2$^+$/ER$^-$ and HER2$^+$/ER$^+$ (Sorlie et al., 2001; Sorlie et al., 2003). Basal like and Her2$^+$ groups have been associated with poor clinical outcomes (Sorlie et al., 2001; 2003; Sotiriou et al., 2003; Nielsen et al .,2004).

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Table 1. Panel of Antibodies Used in the Study

Antibody	Clone	Dilution	Company
ER	SP1	1:100	Thermo Fisher Scientific
PR	PgR1294	1:100	Dakocytomation
Her2/neu	SP3	1: 50	Thermo Fisher Scientific
Cytokeratin 5/6	D5/16B4	1: 50	Dakocytomation
E-cadherin	NCH-38	1:100	Dakocytomation

ER, Estrogen receptor; PR, Progesterone receptor

Nielsen et al proposed an immunohistochemical (IHC) panel, comprising ER, epidermal growth factor receptor (EGFR), HER2 and cytokeratin 5/6 (CK5/6), which could be used to identify breast carcinomas with a basal-like phenotype as defined by cDNA microarrays (Nielsen et al.,2004).

This study was undertaken in attempt to sub classify breast cancers in patients from India using IHC staining proposed by several studies (Sorlie et al., 2001; Sorlie et al., 2003; Nielsen et al.,2004). Our data includes 107 consecutive cases of invasive breast carcinoma from the regional referral health center in central India that serves a population of 2.4 million. We used IHC staining for ER, PR, Her-2, and CK5/6 to identify intrinsic subtypes using formalin-fixed, paraffin-embedded tumor blocks. Tumors were classified to the following subtypes: luminal A (ER^+ and/or PR^+, HER2), luminal B (ER^+ and/or PR+, $HER2^+$), $HER2^+$ subtype ($HER2^+$, ER/PR^+/), and basal-like (ER/ PR , HER2 , cytokeratin $5/6^+$). Tumors that were negative for all 5 markers were considered unclassified. Associations between tumor subtypes and tumor characteristics were examined.

Materials and Methods

Study participants

Tumor samples from 107 consecutive patients with primary breast carcinoma were selected from January 2006 to January 2007 from the department of surgical pathology of Sri Aurobindo Institute of Medical Sciences (SAIMS), Indore. Detailed, clinical and histopathological data of all the cases was available.

Immunohistochemistry

Sections from formalin-fixed, paraffin embedded tumors were cut and mounted on slides. After deparaffinization in xylene, slides were rehydrated through graded series of alcohol and placed in tris buffer. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase and methanol. Commercially available antibodies to ER, PR, Her2/neu, Cytokeratin 5/

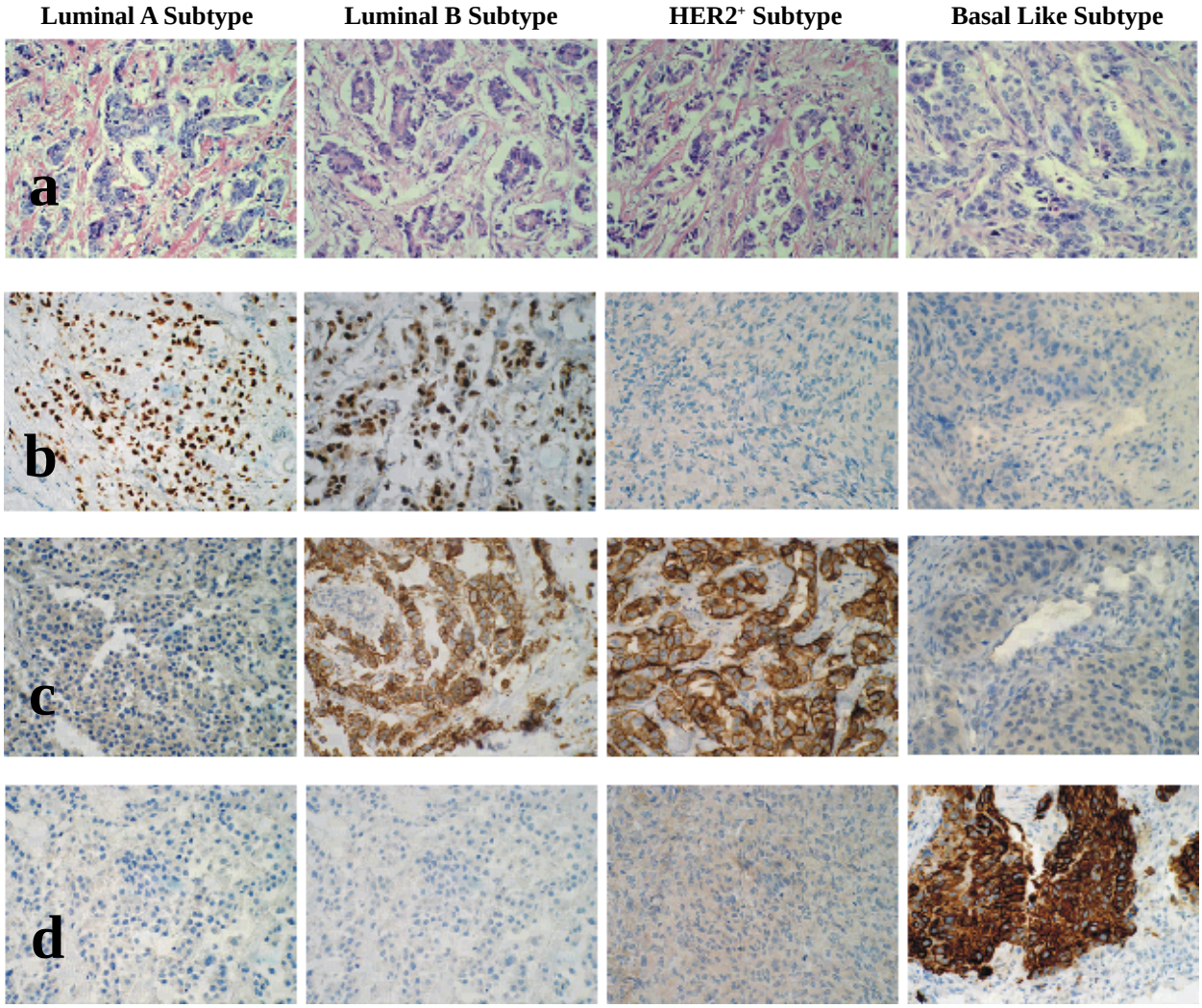

Figure 1. Microphotographs showing Morphology and IHC Results for ER, PR, Her2 and CK 5/6. Rows a)Hematoxylin and Eosin; b) IHC stain, ER/PR; c) IHC stain,Her2; d) IHC stain, CK 5/6; X 400

Table 2. Characteristics of Patients with Immunohistochemical Marker Data

Variables		All cases (n-107) 100%	Luminal A (n-40) 37.3%	Luminal B (n-12) 1.2%	Her2+ (n-31) 29%	Basal Like (n-8) 7.5%	Unclassified (n-16) 15%	p value
Surgery	Partial	37 (35.0)	15 (38.0)	2 (17.0)	12 (39.0)	4 (50.0)	4 (25.0)	0.440
	Total	70 (65.0)	25 (62.0)	10 (83.0)	19 (61.0)	4 (50.0)	12 (75.0)	
Age	Mean	49.4	53.1	47.4	47.3	51.6	44.3	<0.050
	Median	48	55	45	48	49.5	45	
	Range	30-95	32-95	45-60	32-70	45-65	30-65	
Laterality	Left	42 (39.0)	18 (45.0)	4 (33.0)	11 (35.0)	3 (38.0)	6 (37.0)	0.830
	Right	65 (61.0)	22 (55.0)	8 (67.0)	20 (65.0)	5 (62.0)	10 (63.0)	
Tumor type	IDC	102 (95.0)	36 (90.0)	12 (100)	31 (100)	7 (88.0)	16 (100)	0.180
	ILC	3 (3.0)	3 (8.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
	IDC & ILC	2 (2.0)	1 (2.0)	0 (0.0)	0 (0.0)	1 (12.0)	0 (0.0)	
Differentiation	Well/Mod	38 (35.0)	24 (60.0)	6 (50.0)	6 (19.0)	2 (25.0)	0 (0.0)	<0.001
	Poor	69 (65.0)	16 (40.0)	6 (50.0)	25 (81.0)	6 (75.0)	16 (100)	
Tumor size	1	11 (10.0)	8 (20.0)	1 (8.0)	0 (0.0)	0 (0.0)	2 (13.0)	0.140
(pT)	2	76 (71.0)	28 (70.0)	8 (67.0)	25 (80.6)	5 (62.0)	10 (62.0)	
	3	20 (19.0)	4 (10.0)	3 (25.0)	6 (19.4)	3 (38.0)	4 (25.0)	
Nodal status	Negative	19 (28.0)	6 (23.0)	4 (44.0)	2 (11.0)	0 (0.0)	8 (67.0)	<0.050
(pN)	Positive	49 (72.0)	20 (77.0)	5 (56.0)	16 (89.0)	4 (100)	4 (33.0)	
	NA	39	14	3	13	4	4	
LVI	Yes	51 (47.7)	20 (50.0)	5 (41.0)	18 (58.0)	4 (50.0)	4 (25.0)	0.290
	No	56 (52.3)	20 (50.0)	7 (59.0)	13 (42.0)	4 (50.0)	12 (75.0)	
Nuclear	Mild/Mod	57 (53.3)	29 (73.0)	7 (50.0)	14 (45.0)	3 (37.5)	4 (25.0)	0.005
pleomorphism	Severe	50 (47.7)	11 (27.0)	7 (50.0)	17 (55.0)	5 (62.5)	12 (75.0)	
Mitotic count	<10/hpf	51 (46.7)	27 (67.0)	8 (67.0)	12 (39.0)	4 (50.0)	0 (0.0)	<0.001
	>10/hpf	56 (52.3)	13 (33.0)	4 (33.0)	19 (61.0)	4 (50.0)	16 (100)	
ER	Positive	44 (41.1)	35 (88.0)	11 (92.0)	0 (0.0)	0 (0.0)	0 (0.0)	<0.001
	Negative	63 (58.9)	5 (12.0)	1 (8.0)	31 (100)	8 (100)	16(100)	
PR	Positive	44 (41.1)	38 (95.0)	10 (83.0)	0 (0.0)	0 (0.0)	0 (0.0)	<0.001
	Negative	63 (58.9)	2 (5.0)	2 (17.0)	31 (100)	8 (100)	16(100)	
Her2	Positive	43 (40.2)	0 (0.0)	12 (100)	31 (100)	0 (0.0)	0 (0.0)	<0.001
	Negative	64 (59.8)	40 (100)	0 (0.0)	0 (0.0)	8 (100)	16 (100)	
Menopausal	Pre	49 (46.0)	16 (40.0)	7 (58.3)	14 (45.1)	2 (25.0)	10 (62.5)	*0.340
status	Post	58 (45.?)	24 (60.0)	5 (41.6)	17 (54.8)	6 (75.0)	6 (37.5)	

ER, Estrogen receptor; PR, Progesterone receptor; LVI, Lymphovascular invasion; NA, Not available; * Fisher exact test

6 and E-cadherin were used in the study (Table 1). After tissue pretreatment including steam antigen retrieval and protein block, slides were incubated with antibody followed by incubation with horse radish peroxidase-conjugated HRP. 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen was used for visualization of the antibody/enzyme complex. Appropriate positive and negative controls were included with each IHC run. All cases were studied for ER, PR and Her2 antibodies. Cases which were triple negative (ER, PR and Her2 negative) were studied for CK5/6 antibody. Cases diagnosed as lobular carcinoma on hematoxylin and eosin (H&E) staining were confirmed using E-cadherine antibody. Staining results were assessed by two of the authors (K.M. & A.A.) on a double headed microscope. A threshold of 10% of positive neoplastic cells was adapted for ER, PR & CK5/6. HER2 was scored according to the guidelines for Herceptest (Jacobs et al., 1999). A case was considered positive for a given marker only when both observers agreed upon its specificity and distribution.

Statistical analysis

Age difference was examined using 1-way analysis of variance (ANOVA). $\chi 2$ (chi-square) was used to compare the following variables: tumor grade, nuclear grade, mitotic index, tumor size, lymphovascular invasion, nodal status and tumor stage. Fisher exact test was used when expected cell counts were less than 5. Odds ratios (ORs) and 95% confidence intervals (CIs) were also calculated wherever applicable to estimate the magnitude of association among breast cancer subtypes.

Results

A total of 107 cases of infiltrating breast carcinoma were included in the study. Tumor characteristics of all breast cancer subtypes are presented in Table 2 and each subtype is represented in Figure 1.

Mean and median age of our study population was 49.4 and 48 respectively (range 30-95). Majority of patients (65.4%) underwent total mastectomy. Infiltrating duct carcinoma (IDC) was the predominant histopathological type (93.3%, n=102). Histopathological evaluation showed a large proportion of patients (64.5%) with poorly differentiated tumors. 32.7% patients had moderately differentiated while only 2.8% patients had well differentiated tumors.

The proportion of breast cancer subtypes in our patient population was as follows: luminal A subtype (37.4%; 40/107), Luminal B subtype (11.1%; 12/107), $Her2^{+}$ (29%; 31/107), basal-like (7.5%; 8/107) and unclassified 15%; 16/107). The number of tumors that are Her2 positive

Table 3. Hormonal Receptor Status of Breast Cancers with Tumor Characteristics

Variables		ER/PR$^+$ (n=52)%	ER/PR$^-$ (n =55)%	p value
Age	Mean	49.4	47.5	<0.050
	Range	32-95	30-75	
Differentiation	Well/Moderate	30 (57.7)	8 (14.5)	<0.001
	Poor	22 (42.3)	47 (85.5)	
Tumor size*	1	9 (17.3)	2 (3.7)	0.250
(pT)	2	36 (69.2)	40 (72.7)	
	3	7 (13.4)	13 (23.6)	
Nodal Status*	Negative	10 (28.5)	10 (29.4)	0.410
(pN)	Positive	25 (71.5)	24 (70.6)	
	NA	17	21	
Her 2	Positive	12 (23.0)	31 (56.4)	<0.001
	Negative	40 (77.0)	24 (43.6)	
Nuclear grade	Mild/Moderate	36 (69.2)	21 (38.2)	<0.010
	Severe	16 (30.8)	34 (61.8)	
Mitotic index	>10/hpf	35 (67.3)	16 (29.1)	<0.001
	<10/hpf	17 (32.7)	39 (70.9)	
LVI	No	27 (51.9)	29 (52.7)	0.300
	Yes	25 (48.1)	26 (47.3)	

*Fisher exact test; ER, Estrogen receptor; PR, Progesterone receptor; NA, Not available; LVI, Lymphovascular invasion

subtype (29%) was more than previously reported. Patients with Luminal A subtype tumors were significantly older than patients with other subtypes (p<.05).

Her2$^+$ and basal-like subtypes were associated with poor differentiation (p<0.05), higher nuclear grade (p<.05) and high mitotic index (p<0.05). Overall, 51.4% patients had ER/PR negative tumors (Table 3). This group of patients was significantly younger than patients with hormone receptor positive tumors (mean age 47.7 vs. 51.7; p<0.05). ER/PR negative tumors were also significantly associated with poor differentiation (p<0.001), high nuclear grade (p<0.01) and high mitotic index (p<0.001). Her2 over-expression was inversely associated with ER/PR expression (p<0.001). Her2 was over-expressed in 56.3 % ER/PR negative tumors while only 23% ER/PR positive tumors showed Her2 over expression.

Compared with luminal A subtype, Her2$^+$ subtype tumors were 6.2 times more likely to be poorly differentiated (p<.01) and 3.2 times more likely to show both high nuclear grade and high mitotic index (p<0.05). (Table 4). Compared with luminal A subtype, basal-like subtype was 10.5 times more likely to be poorly differentiated (p<0.05), 7.9 times more likely to have high nuclear grade (p<0.05) and 6.2 times more likely to have high mitotic index (p<0.05).

In our study, 63.5% of cases presented in stage III (AJCC). 72.1% (49/68) of patients that underwent axillary dissection had positive lymph nodes. Patients with Her2$^+$ subtype tumors showed 88.8 % (16/18) nodal metastasis and patients with basal like subtype tumors showed 100 %(4/4) nodal metastasis. Tumor size and lymphovascular invasion (LVI) were not significantly associated with any of the tumor IHC subtypes.

Discussion

Breast cancer is a heterogeneous disease composed of growing number of recognized biological subtypes. Prognostic indicators based on currently available clinical and histopathologic variables such as tumor size, tumor grade, lymph node status and hormone receptor status already exist and are used to predict a patient's clinical outcome in certain situations (Galea et al., 1992; Eifel et al., 2001; Goldhirsch et al., 2003; Olivotto et al., 2005). However, these indicators are still inadequate in that within a given patient population with a specific predicted risk of recurrence, there are always patients whose actual clinical outcome doesn't match that predicted by the indicator. Therefore attempts have been made to use molecular profiling to create more accurate prognostic indicators to address these issues (Sorlie et al., 2001; Ahr et al., 2002; Iwao et al., 2002; Van et al., 2002; Van't et al., 2002; Huang et al., 2003; Sorlie et al., 2003; Sotiriou et al., 2003; Ma et al., 2004; Paiket al., 2004; Foekens et al., 2006).

cDNA microarray studies are reshaping breast cancer taxonomy. Sorlie et al. first identified distinct molecular subtypes of breast cancer using gene expression pattern in 65 surgical breast specimens (Sorlie et al., 2001). It has been suggested that breast cancers can be classified according to their gene expression profiles into four main groups: luminal (A and B), HER2$^+$, basal-like, and normal breast-like breast carcinomas (Perou et al.,2000; Sorlie et al.,2001; Van et al., 2002 ; Sorlie et al.,2003; Sotiriou et al.,2003 ; Nielsen et al.,2004). Most importantly, these groups may have prognostic and predictive implications

Table 4. Odds Ratios with 95% Confidence Intervals for Patient and Tumor Characteristics by Breast Cancer Subtypes

Characteristics	Her2$^+$ n=31	p value	Basal like n=8	p value	Luminal B n=12	p value	Unclassified n=16	p value
Menopausal status								
post v pre	1.2 (0.4-3.1)	0.34*	0.5 (0.0-2.7)	0.51*	2.1 (0.5-7.7)	0.28*	2.5 (0.7-8.2)	0.15*
Histopathological differentiation grade								
poor v well/mod	6.6 (2.0-18.6)	<0.01	10.5 (1.1-93.7)	<0.05	1.5 (0.4-5.8)	0.42*	6.8 (1.9-24.4)	<0.001
Nuclear Grade								
marked v slight	3.2 (1.8.6)	<0.05	7.9 (1.3-45.2)	<0.05	1.8 (0.4-7.2)	0.36*	7.9 (2.0-29.8)	<0.01
Mitotic Index								
>10/hpf v <10/hpf	3.2 (1.2-8.7)	<0.05	6.2 (1.1-35.2)	<0.05	1.0 (0.2-4.0)	0.55*	14.2 (4.5-44.9)	<0.001
Tumor stage								
III+IV v I+II	0.63 (0.1-3.2)	0.56	3.6 (0.1-95.0)	0.94*	0.15 (0.0-0.8)	0.45*	3.0 (0.8-10.9)	0.11*
LVI present v absent	0.72 (0.2-1.8)	0.30*	1.0 (0.2-4.5)	0.60*	1.4 (0.3-5.1)	0.45*	3.0 (0.8-10.9)	0.11*

hpf, High power field; LVI, Lymphovascular invasion; * Fisher exact test

Table 5. Immunohistochemical Panel for Breast Cancer Classification as Defined by Nielsen

Group	HER2	ER	CK5/6-EGFR
HER2	+	Any	Any
Luminal	-	+	Any
Basal-like	-	-	+
Undetermined	-	-	-

CK, Cytokeratin; EGFR, epidermal growth factor receptor; ER, Estrogen receptor

(Sorlie et al.,2001; Van et al.,2002 ; Sorlie et al.,2003; Sotiriou et al.,2003 ; Abd El-Rehim et al.,2004 ; Nielsen et al.,2004 ; Abd El-Rehim et al., 2005).

The aim of this study was to identify the various intrinsic subtypes of invasive breast carcinoma, in a district of Central India, Indore. We used IHC surrogates for categorization and determined, for the first time in this population, the prevalence of these subtypes. Although IHC-based assays do not provide as much biological insight into tumor biology as mRNA-based assays containing thousands of genes, this IHC assay allowed classification of tumors into categories that have demonstrated associations between intrinsic subtypes and their prognosis and predictive value (Olopade et al., 2001; Sorlie et al., 2001; Foulkes et al., 2003; Sorlie et al., 2003; Abd El-Rehim et al., 2004; Nielsen et al., 2004). The IHC-based classification system also allows analyses of subtypes to be conducted in patient populations where fresh tissue is not available.

Nielsen et al. proposed an IHC panel, comprising ER, EGFR, HER2 and CK5/ 6, to classify breast cancers and identify breast carcinomas with a basal-like phenotype as defined by cDNA microarrays (Table 5) (Nielsen et al.,2004). Other studies using selected IHC stains have also achieved similar stratifications of tumors according to clinical outcomes, suggesting that this molecular classification is robust (Nielsen et al., 2004; Carey et al.,2006). Based on these studies we used a panel of ER, PR, Her2 and CK5/6 for classification of invasive breast carcinoma.

Subsequent to Nielsen a number of studies have been done in an attempt to classify breast cancer on the basis of IHC surrogates and identified the various subtypes. A summary of these studies along with the findings in our study is presented in Table 6 (Nielsen et al., 2004; Carey et al.,2006; Fan et al.,2006; Carey et al., 2007; Livasy et al., 2007 ; Yang et al., 2007).

Consistent with prior reports, our study shows that luminal breast cancer subtypes were more common and less aggressive than HER2 and basal-like subtypes (Sorlie et al., 2001; Van et al., 2002; Sorlie et al., 2003; Sotiriou et al., 2003; Nielsen et al.,2004). However, there were more breast cancers with $Her2^+$ subtype in our patient population (29%) than previously published (8 to 23%).

Table 6. Comparative Findings of Different Studies on Breast Cancer Subtypes

Author	Total	Her2+	Luminal (A+ B)	Basal like	Un classified
Neilson (2004)	663	23%	40%	15%	22%
Carey (2006)	496	7%	67% (51+16)	20%	6.2%
Carey (2007)	107	11%	62%	32%	0%
Livasy (2006)	245	16%	84% (61+23)	8%	6%
Fan (2006)	295	12%	60% (42+19)	18%	9.8%
Yang (2007)	804	8%	75% (69+ 6)	12%	6%
Present study	107	29%	49% (37+11)	7.5%	15%

Studies have shown that basal-like subtype has been associated with poor clinical outcomes (Sorlie et al., 2003; Sotiriou et al., 2003; Nielsen et al., 2004). In our study basal like subtype showed a prevalence of 8% and exhibited aggressive features including, high nuclear grade and high mitotic index. Our study showed that the majority of cases (64%) presented in stage III (AJCC). This finding is similar to the data reported by the National Cancer Registry of India (NCRI), which reported 70% of all patients seeking treatment for breast cancer, present with locally advanced disease (NCRI 2001). This is most likely attributed to the lack of screening mammogram in India , where patients seek medical attention at an advanced stage of breast cancer.

In conclusion, our data shows higher proportion of patients with poorly differentiated, node positive cancer with LVI than patients in developed countries. Our patients were also more likely to be treated with total mastectomy (65.4%). We observed a lower number of basal-like and a higher proportion of $Her2^+/ER^-$ subtypes than previously reported. Patients with $Her2^+/ER^-$ tumors were significantly associated with poor differentiation (P= 0.0005) than the other subtypes combined. The increasing incidence of breast cancer and presentation of patients with advanced disease, in the Indian population, warrant more focus on early detection and preventive measures.

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