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Identification of different subtypes of breast cancer using tissue microarray

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Abstract

Breast cancer may be classified into luminal A, luminal B, HER2+/ER-, basal-like and normal-like subtypes based on gene expression profiling or immunohistochemical (IHC) characteristics. The main aim of the present study was to classify breast cancer into molecular subtypes based on immunohistochemistry findings and correlate the subtypes with clinicopathological factors. Two hundred and seventeen primary breast carcinomas tumor tissues were immunostained for ER, PR, HER2, CK5/6, EGFR, CK8/18, p53 and Ki67 using tissue microarray technique. All subtypes were significantly associated with Malay ethnic background (p=0.035) compared to other racial origins. The most common subtypes of breast cancers were luminal A and was significantly associated with low histological grade (p<0.000) and p53 negativity (p=0.003) compared to HER2+/ER-, basal-like and normal-like subtypes with high histological grade (p<0.000) and p53 positivity (p=0.003). Luminal B subtype had the smallest mean tumor size (p=0.009) and also the highest mean number of lymph nodes positive (p=0.032) compared to other subtypes. All markers except EGFR and Ki67 were significantly associated with the subtypes. The most common histological type was infiltrating ductal carcinoma, NOS. Majority of basal-like subtype showed comedo-type necrosis (68.8%) and infiltrative margin (81.3%). Our studies suggest that IHC can be used to identify the different subtypes of breast cancer and all subtypes were significantly associated with race, mean tumor size, mean number of lymph node positive, histological grade and all immunohistochemical markers except EGFR and Ki67.

Keywords: immunohistochemistry, breast cancer, estrogen receptor, progesterone receptor, HER2, keratins.

Introduction

Breast cancer is a heterogeneous disease such that they may have different prognoses and respond to therapy differently despite similarities in histological types, grade and stage. These differences are not well understood but are possibly due to the differences in the cell of origin. Normal human mammary gland epithelium is characterized by ductal glandular or luminal, basal myoepithelial and mammary gland stem/progenitor cells [1]. Breast cancers of glandular epithelial immunophenotype suggest that these neoplastic cells are derived from late stage of the glandular epithelial differentiation.

Gene expression profiling in breast cancer has been successfully used to classify breast cancer into pathologically distinct subtypes [2]. For example, cDNA microarray studies on breast carcinoma have shown that breast cancers can be divided into expressing ER (ER+) luminal subtype and non expressing ER (ER-) tumors which in turn encompass three subgroups; HER2+, basal-like and normal-like [2, 3]. However, the application of cDNA microarrays in the clinical setting is still limited due to financial concerns and the few samples that it can test at one time. On the other hand, tissue microarray (TMA) and immunohistochemistry can be used to test few markers on a large number of samples [4]. Given the limitations of molecular classification of breast cancer based on gene expression profiling, an immunohistochemistry based molecular classification has been proposed [3, 5, 6]. Immunohistochemistry demonstrated good and acceptable surrogate of the gene analysis [3, 7, 8].

Previous immunohistochemical based molecular profiling studies on breast cancer have found that basal-like cancers tend to be associated with triple negative phenotype (immunohistochemically defined as ER, PR

and HER2 negative) [6, 9]. In addition, Tan DSP *et al.* (2008) have found that the majority of triple negative tumor markers also expressed known basal markers such as CK5/6, CK14 and CK17, indicating the tumor heterogeneity of the triple negative phenotype tumors [10]. Basal CK5/6 and CK14 and other molecular markers such as vimentin and p63 were also used to define basal-like subtype by expressing one or a combination of these markers [5, 11, 12]. These immunohistochemical studies paralleled with gene expression studies of basal-like subtypes, which showed high gene expression of CK5, CK17, vimentin, EGFR and c-kit [2, 3, 13]. Therefore, findings combining triple negative tumors and cytokeratin expression of tumor subtypes derived from the data of gene expression profiling can be used as an approach to form an IHC based classification [3].

In 2006, breast cancer was the most common cancer in Malaysian women and was also the leading cause of cancer regardless of sex in Peninsular Malaysia [14]. The main aim of the present study was to examine and identify the different subtypes of breast carcinomas in Malaysian population by immunohistochemistry using tissue microarray technique.

Materials and Methods

Patients

This study was approved by Ethical Committee of Universiti Kebangsaan Malaysia Medical Centre. A total of two hundred and seventeen patients diagnosed with invasive breast carcinoma were recruited from two hospitals, Hospital Universiti Kebangsaan Malaysia and Hospital Putrajaya comprising of 213 (98.2%) females and four males. Clinical information was retrieved from the medical records. Breast cancers were classified according to the *World Health Organization* (WHO, 2003) [15] while histological grading and staging were performed according to Modified Bloom-Richardson classification [16] and *American Joint Committee on Cancer* (AJCC) [17], respectively.

Tissue microarray construction

A tissue microarray of 217 breast carcinomas was constructed into four blocks from which a total of 31 tissue array blocks were built. These Hematoxylin–Eosin (H&E) stained sections were made from each original block to define representative tumor regions and to determine the spots that are suitable before encircling with a marker [18].

Guided by marked H&E stained slides, cores from the selected area of the donor blocks were punched with 0.6 mm diameter needle using manual MTABooster from Alphelys (Plaisir, France) and inserted into new paraffin block (recipient block) with 1.8 mm spacing between the cores at defined array coordinates. In total, four tissue cores were sampled from the donor blocks. Two tissue cores were taken from the centre of the tumor donor block with the remaining cores taken from the periphery of the tumor. Following completion of the TMA block, the block was then heated at 60^0C for approximately 5 minutes, which was able to melt the wax thus closing the small gap between the inserted cores.

Immunohistochemical staining

The immunohistochemical staining was performed on tissue microarray as described previously with minor modifications [19]. A detailed summary of primary antibodies (biomarkers) used for immunohistochemistry (IHC) including their clone, dilution and antigen retrieval were shown in Table 1.

Table 1 – ***The primary antibodies used for immunohistochemistry (IHC) staining***

Primary antibodies (biomarkers)	Clone / Source	Dilution	Antigenic retrieval
Estrogen receptor (ER)	1D5 / DAKO	1:75	Tris/EDTA, pH 9 (water bath, 40 minutes)
Progesterone receptor (PR)	PgR 636 / DAKO	1:75	Citrate buffer, pH 6 (water bath, 40 minutes)
HER2 (c-erbB-2)	SP3 / Neomarker	1:350	Citrate buffer, pH 6 (water bath, 40 minutes)
Cytokeratin 5/6 (CK5/6)	D5/16 B4 / DAKO	1:75	Tris/EDTA, pH 9 (water bath, 40 minutes)
Epidermal growth factor receptor (EGFR)	111.6 / Thermo Scientific	1:50	Proteanase K (room temperature, 5 minutes)
Cytokeratin 8/18 (CK8/18)	5D3 / Neomarker	1:75	Pepsin (room temperature, 5 minutes)
P53	DO7 / DAKO	1:150	Tris/EDTA, pH 9 (water bath, 40 minutes)
Ki67	MIB1 / DAKO	1:75	Citrate buffer (pt), pH 6 (water bath, 40 minutes)

Positive and negative controls were included for each immunohistochemical run. The positive control slides were prepared from tissues of breast cancer (ER, PR, HER2, and CK8/18), adenocarcinoma of the colon (p53), tonsil (CK5/6 and Ki67) and placenta (EGFR). The negative control slides were prepared from the same tissue blocks used for positive controls but TBS buffer was used instead of the primary antibody.

Evaluation of immunohistochemistry

The expressions of all markers were evaluated by two independent pathologists. ER (estrogen receptor), PR (progesterone receptor), p53 and Ki67 stains were considered positive if immunostaining was seen in more (>) than 10% of tumor nuclei [5, 11, 13].

In this study, a positive staining (3+) for HER2 corresponds to strong complete staining in >30% of tumor cells whereas weak to moderate complete staining in >10% cells was scored as equivocal (2+) and neither staining nor faint incomplete staining was scored as negative (0 and 1+) [20]. Cases scoring 2+ were subsequently assessed by fluorescence *in situ* hybridization (FISH) [6].

EGFR staining was considered positive if at least (≥) 10% membrane staining of tumor cells was observed [11]. A positive score for CK5/6 and CK8/18 was

recorded if any cytoplasmic and/or membrane staining of any invasive malignant cells was present [21].

Fluorescence *in situ* hybridization (FISH) technique

FISH was performed using PathVysis HER2 DNA Probe kit (Abbott Molecular, Canada) according to the manufacturer's instructions and with minor modification. Tissue whole section were cut into 3 μm thick and adhered on silanized slide. The slides were baked overnight at 60^0C, deparaffinized and pre-treatment with Skip-dewax (1:10, Dako, Glostrup, UK) for one hour. Then, slides were washed in distilled water for 2 minutes each before incubation in Protease (Vysis Abbott Molecular, Canada) for 50 minutes at 37^0C with agitation. Slides were washed twice in 2% sodium chloride/sodium citrate (SSC) at room temperature, 5 minutes each, and were then allowed to air dry. Slides were incubated with DNA probe (LSI HER2/neu SpectrumOrange/CEP17 SpectrumGreen, PathVysis, Abbott Molecular, Canada) overnight at 37^0C after denaturing for 15 minutes at 90^0C. After hybridization, the slides were washed for 2 minutes with agitation in 0.4% SSC / 0.3% NP40 at 74^0C followed by ×2 SSC 0.1% NP40 at room temperature. The slides were allowed to air dry before they were counterstained with diamidino-2'-phenylindole (DAPI, PathVysis HER2 DNA Probe kit, Abbott Molecular, Canada) and stored at 4^0C in the dark.

Evaluation of FISH

The slides were evaluated using fluorescence microscope with Applied Spectral Imaging (ASI) software and examined by an investigator and a pathologist. The number of LSI HER2/neu in orange signal and CEP17 in green signal per nucleus was recorded. For each case, 60 non-overlapping nuclei of invasive carcinoma cells were counted and scored as amplified when the mean ratio between LSI HER2/neu and CEP17 was greater than 2 [6].

Definition of breast cancer subtypes

The tumors were classified into molecular subtypes and according to immunohistochemical expression profiles of ER, HER2, EGFR and CK5/6. The cases were classified into molecular subtypes of luminal A (ER+ and/or PR+, HER2-), luminal B (ER+ and/or PR+, HER2+), HER2+/ER- (ER-, PR-, HER2+), basal-like (ER-, PR-, HER2-, CK5/6+ and/or EGFR+) and normal-like tumors (negative for all five markers) [3, 8]. The expression of CK8/18, p53 and Ki67 were evaluated on all subtypes.

The basal-like subtypes were examined on whole paraffin sections of breast carcinomas stained by H&E for pathologic evaluation. All samples were examined by two independent pathologists.

Statistical analysis

All statistical analyses were performed using SPSS version 12. Analysis chi-square Pearson was used to investigate the association between categorical variables. The patients' mean age, tumor size and mean numbers of lymph nodes positive were evaluated by Kruskal–Wallis. A p-value of less than 0.05 ($p<0.05$) was considered as statistically significant.

Results

Patients, tumor subtypes and tumor characteristics

Table 2 showed the tumor subtypes and demographic data breast cancer patients and there was a total of 217 cases.

Table 2 – ***Tumor subtypes and demographic data of patients with invasive breast carcinoma***

	Total 217 (100%)	Luminal A 125 (57.6%)	Luminal B 15 (6.9%)	HER-2+/ER- 31 (14.3%)	Basal-like 16 (7.4%)	Normal-like 30 (13.8%)	*P*-value
Age at diagnosis [years]:							
Mean±SD	53.1±11.5	55.1±12	48.2±10.7	52.6±9.7	48.3±11.4	50.1±9.7	0.189
(range)	(27–87)	(31–87)	(31–66)	(36–74)	(27–66)	(38–73)	
<50	100 (46.1%)	49 (39.2%)	9 (60%)	14 (45.2%)	9 (56.3%)	19 (63.3%)	
>50	117 (53.9%)	76 (60.8%)	6 (40%)	17 (54.8%)	7 (43.7%)	11 (36.7%)	
Menopause status:							
Pre	77 (41.8%)	41 (37.6%)	8 (53.3%)	11 (44%)	6 (46.2%)	11 (50%)	0.669
Post	107 (58.2%)	68(62.4%)	7 (46.7%)	14 (56%)	7 (53.8%)	11 (50%)	
Missing	33	16	0	6	3	8	
Race:							
Malay	128 (59%)	62 (49.6%)	13 (86.7%)	21 (67.7%)	11 (68.8%)	21 (70%)	0.035
Chinese	69 (31.8%)	51 (40.8%)	1 (6.7%)	8 (25.8%)	4 (25%)	5 (16.7%)	
Others	20 (9.2%)	12 (9.6%)	1 (6.7%)	2 (6.5%)	1 (6.3%)	4 (13.3%)	
Tumor size [cm]:							
Mean±SD	4.2±3.1	3.6±2.3	3.4±2.5	6.2±5.1	4.7±3.3	4.6±2.6	
(range)	(0.5–24)	(0.5–15)	(1–11)	(1.4–24)	(1.7–15)	(0.6–10.5)	
0.1–2	52 (24%)	36 (28.8%)	5 (33.3%)	5 (16.1%)	3 (18.8%)	3 (10%)	0.009
2.1–5	116 (53.5%)	69 (55.2%)	8 (53.3%)	14 (50%)	8 (50%)	17 (56.7%)	0.069
>5	49 (22.6%)	20 (16%)	2 (13.3%)	12 (38.7%)	5 (31.3%)	10 (33.3%)	
Lymph node (LN) [cm]:							
Mean±SD	3.5±5.7	2.6±4.7	5.4±6.5	4.9±6.7	4.7±7.7	4.0±6.7	
(range)	(0–31)	(0–21)	(0–17)	(0–31)	(0–23)	(0–23)	
Negative	100 (46.1%)	66 (52.8%)	4 (26.7%)	9 (29%)	8 (50%)	13 (43.3%)	0.032
Positive	117 (53.9%)	59 (47.2%)	11 (73.3%)	22 (71%)	8 (50%)	17 (56.7%)	0.079

	Total 217 (100%)	Luminal A 125 (57.6%)	Luminal B 15 (6.9%)	HER-2+/ER- 31 (14.3%)	Basal-like 16 (7.4%)	Normal-like 30 (13.8%)	*P*-value
Histological types:							
IDC, NOS	186 (85.7%)	104 (83.2%)	13(86.7%)	31 (100%)	13 (81.3%)	25 (83.3%)	
ILC	11 (5.1%)	7 (5.6%)	1(6.7%)	0	0	3 (10.3%)	
Mucinous Ca	6 (2.8%)	6 (4.8%)	0	0	0	0	0.067
Cribiform Ca	4 (1.8%)	4 (3.2%)	0	0	0	0	
Mixed Ca	5 (2.3%)	3 (2.4%)	0	0	1 (6.3%)	1 (3.3%)	
Metaplastic Ca	3 (1.4%)	0	0	0	2 (12.5%)	1 (3.3%)	
IP Ca	2 (0.9%)	1 (0.8%)	1 (6.7%)	0	0	0	
Grade:							
1	57 (26.3%)	46 (36.8%)	2 (13.3%)	3 (9.7%)	1 (6.3%)	5 (16.7%)	0.000
2	96 (44.2%)	58 (46.4%)	9 (60%)	13 (41.9%)	6 (37.5%)	10 (33.3%)	
3	64 (29.5%)	21 (16.8%)	4 (26.7%)	15 (48.4%)	9 (56.3%)	15 (50%)	
Recurrence:							
Yes	23 (10.6%)	13 (10.4%)	4 (26.7%)	1 (3.2%)	2 (12.5%)	3 (10.1%)	0.242
No	194 (89.4%)	112 (89.6%)	11 (73.3%)	30 (96.8%)	14 (87.5%)	27 (90%)	
Stage:							
I	16 (7.4%)	12 (9.6%)	1 (6.7%)	0	0	3 (10%)	
II	89 (41%)	55 (44%)	7 (46.7%)	10 (3.2.3%)	7 (43.8%)	10 (33.3%)	0.212
III	61 (28.1%)	34 (27.2%)	4 (26.7%)	13 (41.9%)	3 (18.8%)	7 (23.3%)	
IV	51 (23.5%)	24 (19.2%)	3 (20%)	8 (25%)	6 (37.5%)	10 (33.3%)	

IDC – infiltrating ductal carcinoma, not otherwise specified (NOS); ILC – infiltrating lobular carcinoma; Ca – carcinoma; Mixed Ca – mixed ductal and lobular carcinoma; IP Ca – invasive papillary carcinoma.

The luminal A (57.6%) was the most common IHC-subtypes, followed in descending order by HER2+/ER- (14.3%), normal-like (13.8%), basal-like (7.4%) and luminal B (6.9%) subtypes. During 93 months of follow up, only 10.6% of patients had recurrence and 13.4% were died.

Chi-square analysis showed a significant relationship between subtypes and race (p=0.035) and histological grade (p<0000) (Table 2). There were no significant relationships between subtypes and age (p=0.189), menopausal status (p=0.669), tumor size (p=0.069), lymph nodes (p=0.079), histological types (p=0.067), recurrence (p=0.242) and stage (p=0.212). By Kruskal–Wallis analysis, there were statistically significant different between subtypes with mean size of tumor (p=0.009) and mean number of lymph nodes positive (p=0.032).

More than 50% of patients were above 50-year-old (117/217; 53.9%) with mean age of diagnosis at 53.1 years, ranging between 27 and 87-year-old. Luminal A had the oldest mean age (55.1 years) and the highest percentage of patients (76/125; 60.8%) above 50-year-old as compared with other subtypes. The mean age of basal-like tumors was 48.3 years with the youngest being 27-year-old. Luminal A (68/109; 62.4%), HER2+/ER- (14/25; 56%) and basal-like (7/13; 53.8%) subtypes were mainly seen in the post-menopausal women.

The ethnic population of Malaysia countries mainly comprise of three groups of racial background: Malays, Chinese and Indians. In this study, the large racial distribution was Malay (128/217; 59%) followed by Chinese (69/217; 31.8%) and others including Indians (20/217; 9.2%). All subtypes were also more common in Malays followed by the Chinese and the Indians except that the incidence of luminal B subtype was similar for both Chinese and other groups (1/15; 6.7%). All subtypes occurred in more than 65% of Malays but the incidence is slightly less than 50% for luminal A. On the contrary, 40.8% (51/125) of Chinese cases were of luminal A subtype, with other IHC-subtypes being less than 30%.

The size of the tumor ranged between 0.5 to 24 cm (mean 4.2±3.1 cm) and the largest mean size of tumor (6.2±5.1 cm; range 1.4–24 cm) was seen in HER2+/ER- subtype (p=0.009). While the smallest mean size of tumor was seen in luminal B subtype (mean 3.4±2.5 cm). The mean number of lymph node positive was significantly largest in luminal B subtype (5.4±6.5; range 0–17) and smallest in luminal A subtype (2.6±4.7; range 0–21; p=0.032).

The most common histological type was infiltrating ductal carcinomas (IDC), not otherwise specified (NOS) comprising 186 (85.7%) cases. Luminal A, luminal B, HER2+/ER-, basal-like and normal-like subtypes consisted of 83.2% (104/125), 86.7% (13/15), 100% (31/31), 81.3% (13/16) and 83.3% (25/30) of IDC, NOS of the cases respectively.

The majority of patients significantly (p<0.000) showed tumor histologic grade 2 (44.2%; 96/217) followed by grades 3 (64/217; 29.5%) and 1 (57/217; 26.3%). 46.4% (58/125) and 60.0% (9/15) of luminal A and luminal B subtypes respectively showed tumor histologic grade 2 while HER2+/ER- (15/31; 48.4%), normal-like (15/30; 50%) and basal-like subtype (9/16; 56.3%) mainly showed tumor histologic grade 3 (p<0.000). More luminal B subtype tumors (4/15; 26.7%) showed tumor histology grade 3 compared to luminal A (21/125; 16.8%).

Among the subtypes, tumor recurrence was mainly seen in luminal B subtype (4/15; 26.7%). Stage I comprised of 7.4% (16/217) of all cases and was seen in all IHC-subtypes except HER2+/ER- and basal-like but this association was not found to be statistically significant (p=0.212).

Immunohistochemical results

The frequency of immunohistochemical expression in different subtypes of invasive breast carcinoma was presented in Table 3.

Table 3 – ***Immunohistochemistry results in different subtypes of invasive breast carcinomas***

	Total 217 (100%)	Luminal A 125 (57.6%)	Luminal B 15 (6.9%)	HER2+/ER- 31 (14.3%)	Basal-like 16 (7.4%)	Normal-like 30 (13.8%)	*P*-value
ER:							
Positive	120 (55.8%)	112 (90.3%)	8 (53.3%)	0	0	0	0.000
Negative	95 (44.2%)	12 (9.7%)	7 (46.7%)	31 (100%)	16 (100%)	29 (100%)	
Missing	2	1	0	0	0	1	
PR:							
Positive	91 (41.9%)	80 (64%)	11 (73.3%)	0	0	0	0.000
Negative	126 (58.1%)	45 (36%)	4 (26.7%)	31 (100%)	16 (100%)	30 (100%)	
HER2:							
Positive	46 (21.2%)	0	15 (100%)	31 (100%)	0	0	0.000
Negative	171 (78.8%)	125 (100%)	0	0	16 (100%)	30 (100%)	
CK5/6:							
Positive	38 (17.8%)	15 (12.2%)	1 (7.1%)	6 (19.4%)	16 (100%)	0	0.000
Negative	176 (82.2%)	108 (87.8%)	13 (92.9%)	25 (80.6%)	0	30 (100%)	
Missing	3	2	1	0	0	0	
EGFR:							
Positive	3 (1.5%)	1 (0.9%)	0	1 (3.4%)	1 (7.7%)	0	0.438
Negative	191 (98.5%)	112 (99.1%)	14 (100%)	28 (96.6%)	12 (92.3%)	25 (100%)	
Missing	23	12	1	2	3	5	
CK8/18:							
Positive	173 (80.5%)	106 (85.5%)	13 (86.7%)	27 (90%)	11 (68.7%)	16 (53.3%)	0.002
Negative	42 (19.5%)	18 (14.5%)	2 (13.3%)	3 (10%)	5 (31.3%)	14 (46.7%)	
Missing	2	1	0	1	0	0	
P53:							
Positive	120 (55.3%)	60 (48%)	12 (80%)	17 (54.8%)	15 (93.8%)	16 (53.3%)	0.003
Negative	97 (44.7%)	65 (52%)	3 (20%)	14 (45.8%)	1 (6.3%)	14 (46.7%)	
Ki67:							
Positive	89 (41.8%)	47 (38.5%)	10 (66.7%)	13 (41.9%)	9 (56.3%)	10 (34.5%)	0.177
Negative	124 (58.2%)	75 (61.5%)	5 (33.3%)	18 (58.1%)	7 (43.7%)	19 (65.5%)	
Missing	4	3	0	0	0	1	

The most frequent positive immunohistochemical expression was observed in ER (55.8%; 120/215) (Figure 1A) and the least frequent positive expression was seen in EGFR (1.5%; 3/194) (Figure 1B).

There was a significant association between ER, PR, HER2, CK5/6, CK8/18 and p53 with all of the tumor subtypes. However, no significant correlation could be found for EGFR and Ki67.

There were 25 (11.5%) cases of HER2 scoring 2+ that were subsequently assessed by fluorescence *in situ* hybridization (FISH) [6].

Twenty-two of 25 (88%) cases were amplified and three were not. The final total cases positive for HER2 was of 21.2% (46/217).

The ER (Figure 1A) was mainly expressed in 90.3% (112/124) in luminal A and 53.3% (8/15) in luminal B of all cases (p<0.000).

While EGFR (Figure 1B) was expressed in 7.7% (1/13) basal-like, 3.4% (1/29) HER2+/ER- and 0.9% (1/113) luminal A.

PR (Figure 1C) was expressed in 64.0% (80/125) in luminal A and 73.3% (11/15) in luminal B subtypes of all cases.

HER2 (Figure 1D) was expressed only in luminal B (100%; 15/15) and HER2+/ER- (100%; 31/31) but not in luminal A, basal-like and normal-like subtypes.

CK5/6 (Figure 1E) was positive in 100% (16/16), 19.4% (6/31), 12.2% (15/123), 7.1% (1/14) of basal-like, HER2+/ER-, luminal A and luminal B subtypes respectively (p<0.000).

CK8/18 (Figure 1F) positive was seen in all subtypes (p=0.009) whereas p53 (Figure 1G) was expressed in 93.8% (15/16), 80% (12/15), 54.8% (17/31), 53.3% (16/30) and 48% (60/125) of basal-like, luminal B, HER2+/ER-, normal-like and luminal A respectively (p=0.003).

Luminal B (Figure 1H) showed high expression of Ki67 (10/15; 66.7%) followed by basal-like (9/16; 56.3%), HER2+/ER- (13/31; 41.9%), luminal A (47/122; 38.5%) and normal-like (10/30; 34.5%) subtypes (p=0.250).

Figure 1I showed negative staining of the breast carcinoma.

Histologic features of basal-like subtype

The most common histological type was infiltrating ductal carcinoma, NOS (81.3%) and most were in grade 3 (56.3%).

Apart from IDC, basal-like subtype tumors also consisted of metaplastic carcinoma (2/16; 12.5%) and mixed ductal and lobular carcinoma (1/16; 6.3%). Majority of basal-like subtypes showed comedo-type necrosis (10/16; 68.8%) (Figure 2A), infiltrative margin (13/16; 81.3%) and scanty to moderate inflammation (8/16; 50%).

Only a few cases demonstrated solid pattern (5/16; 31.3%), pushing margin (3/16; 18.8%) (Figure 2B), central acellular scar (1/16; 6.3%) (Figure 2C) and squamous cell differentiation (2/16; 12.5%) (Figure 2D).

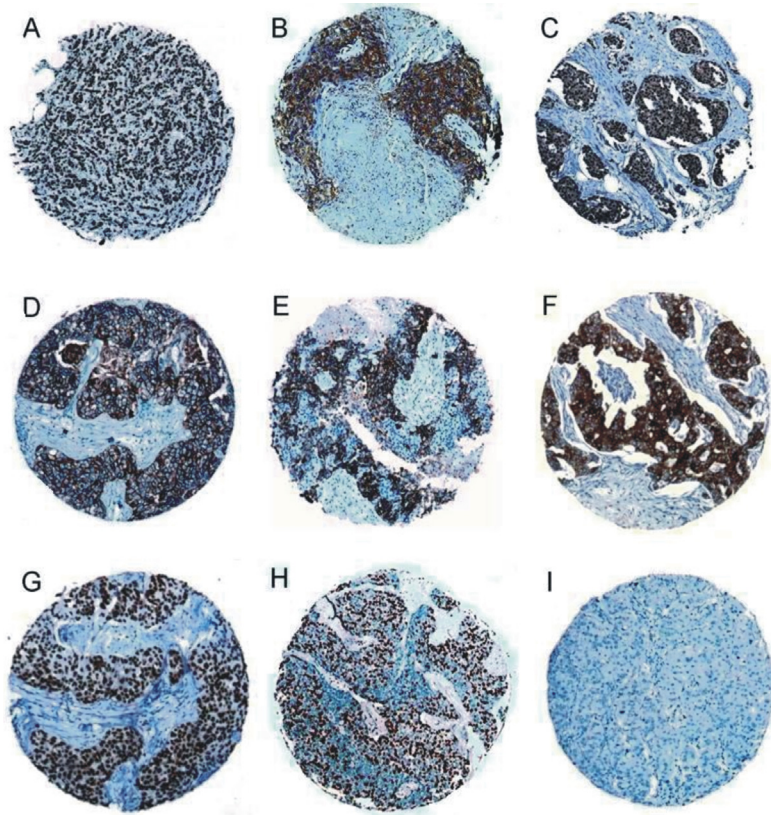

Figure 1 – ***Representative of immunostaining of molecular markers in breast carcinoma using tissue microarray. (A) ER nuclear (brown) immunostaining; (B) EGFR (brown) membranous immunostaining; (C) PR nuclear (brown) immunostaining; (D) HER2 membranous (brown) immunostaining was scored 3+; (E) CK5/6 membranous (brown) immunostaining; (F) CK8/18 membranous (brown) staining; (G) p53 nuclear (brown) immunostaining (H) Ki67 nuclear (brown) immunostaining, and (I) Negative staining (original magnification, ×40).***

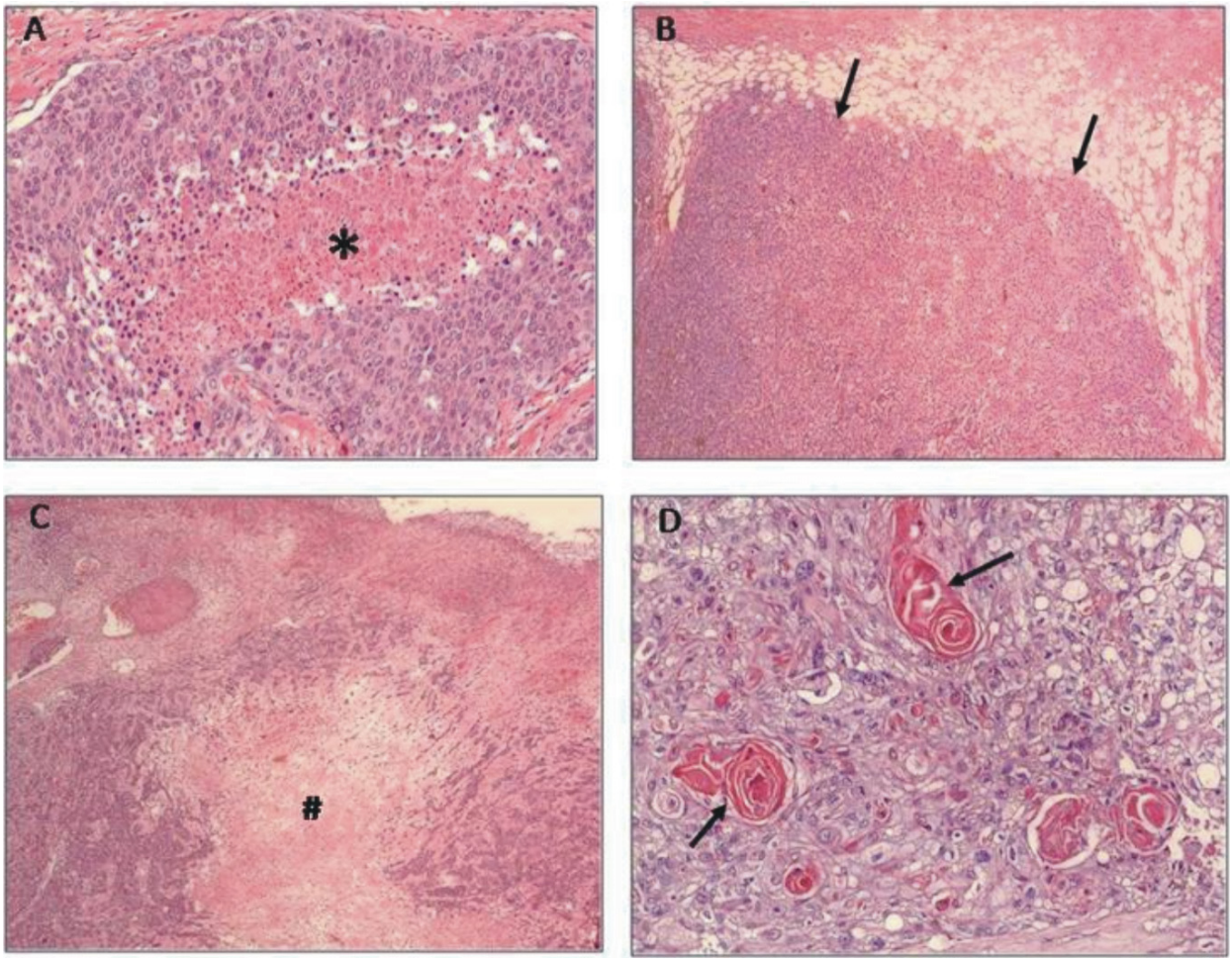

Figure 2 – ***Basal breast carcinomas showing (A) comedo-type necrosis (*), (B) pushing non infiltrative growth margin (→), (C) the characteristic central, acellular 'scar' (#), and (D) squamous cell differentiation (→). Original magnifications are ×100 (A and D) and ×40 (B and C) (Hematoxylin & Eosin staining).***

Discussion

In this present study, luminal A (57.6%) was the most common IHC-subtypes and fall within the range of other studies from 27% up to 73.4% whereas other subtypes were between 5.2% to 32% [9, 13, 22, 23].

We have found significant correlation between breast carcinoma subtypes with ethnicity and histological grade. However, there was no significant association between breast carcinoma subtypes with age, menopausal status, tumor size, lymph nodes status and stage consistent with that previously reported [9, 24, 25].

We have also found that luminal A subtypes of breast cancer were usually observed in patients older than 50-year-old even though it was not significantly associated. On the other hand, 35.5% of patients was found to be 70 years and above [23] and had occurred in postmenopausal women [8, 26].

The Carolina Breast Cancer Study showed that the luminal A subtype was more frequent among post menopausal African American (59%) or non-African American (54%) women [8]. We, on the other hand have found that luminal B subtype was the most frequent among Malay patients regardless of menopausal status.

Our study have also found that the mean number and percentages of positive lymph nodes were high in HER2+/ER- and/or luminal B subtypes, similar to that previously reported [8, 11, 22, 25]. In keeping with previous studies [6, 8, 23, 25], we had also observed the low percentage of positive lymph nodes seen in basal-like subtype cancers. Foulkes WD *et al.* (2004) have found that CK5/6-positive cancers were less likely to be node positive when the tumor is large in size, suggesting a preferred route of metastatic spread for basal cancers [27].

Prior to immunohistochemistry and expression profiling, breast cancer was classified mainly by histology and grade [26]. In this study, we have shown that all cases of invasive ductal carcinoma also displayed HER2+/ER-, suggesting that breast cancer could be classified according to the expression of immunohistochemistry. The two of three (66.7%) cases of metaplastic carcinoma showed basal like subtype features in our study were also similar to previous reports by Kim MJ *et al.* (2006) [11].

We had shown, similar to other studies, that the majority of histological low-grade (1 and 2) tumors also displayed features of luminal A and B, and that high-grade (3) tumors were mainly of HER2+/ER- and basal-like subtypes [8, 22, 23, 25, 26]. Therefore, the features for all subtypes were in accordance with the multistep model for breast cancer progression proposed by Simpson PT *et al.* (2005) which was based on findings that tumors with hormone receptor-negative and frequently positive for either HER2 or basal markers usually progressed towards histological grade 3 [28].

In keeping with another study [23], we had also found that basal-like and HER2+/ER- subtypes were observed in stage III and IV tumors, although this was not found to be significant.

Based on patterns of protein expression, luminal subtypes are classified into luminal A and luminal B in which both express ER, PR, BCL2, CK8 and CK8/18 [13, 29, 30]. Both luminal subtypes may also show low expression of basal molecular markers (CK5, CK5/6 and CK17) as well as other molecular markers (EGFR and vimentin) [13, 30].

Compared to luminal A, luminal B subtypes of breast cancer show less expression of ER and PR [13, 29, 30]. Because luminal B subtype also expresses genes associated with HER2 such as *ERBB2* and *GRB7* [31], HER2 has been used to distinguish luminal A and luminal B. However, HER2 positivity was only seen in 30% of luminal B subtype tumors, indicating that HER2 alone was not sufficiently sensitive in identifying this type of tumors [31]. Luminal B showed high expression of Ki67 compared to luminal A but was not significant in this study. Cheang MCU *et al.* (2009) found that Ki67 together with ER, PR and HER2 could be used to distinguish luminal A and luminal B [31].

HER2+/ER- and basal-like subtypes share similar protein profiles with the exception that HER2 was shown to be expressed by HER2+/ER- subtype and those basal molecular markers such as CK5, CK17, c-kit, EGFR and vimentin were expressed by basal-like subtype tumors [13, 30]. In addition, HER2+/ER- and basal-like subtypes showed either less expression or negative for ER and PR [13, 30] and this was supported by findings of cDNA-microarray studies [2, 3].

This study showed positive frequency of immuno-histochemical expression of HER2 was 21.2% (46/217) of the cases and was within the range of other studies [3, 6, 11]. Past study concluded that HER2 status determination was most efficient by using immunohistochemistry as a method of choice, with FISH performed in cases with moderate (2+) staining [32]. In the present study, 25 (11.5%) tumors were scored 2+ by immunohisto-chemistry; of these 88% were associated with *HER2/neu* gene amplification by FISH. This study employed an IHC staining of 3+ (uniform, intense membrane staining of 30% of invasive tumor cells) that could explain this high percentage [20]. An earlier study had showed thirteen per cent of tumors were IHC 2+ and overall 48% of these were FISH positive but this proportion varied markedly between the centers [33].

Basal molecular markers were not only observed in basal-like subtypes [11, 12, 29, 34] but also in other subtypes with the exception of normal-like. In this study, there were more cases of HER2+/ER- subtypes with positive CK5/6 compare to luminal subtype, consistent with a recent finding [34]. Banerjee S *et al.* (2006) found that basal-like subtypes had expressed ER, PR and HER2 in 18.4%, 20.4% and 8.2% of cases, respectively [5]. A study by Laakso M *et al.* (2006) found that two subtypes, which were uniformly positive type (basal) and a partially positive type (basoluminal), could be distinguished based on basal cytokeratin expression [35]. Both basal and basoluminal subtypes showed high-grade histology and are hormone receptor negative [35]. However, expression of Ki67, vimentin and c-kit were more frequently expressed in basal tumors whereas amplification of HER2 was more characteristic of the basoluminal subgroup. Therefore,

in view of these previous observations, we believe that the 19.4% of HER2+/ER- subtype tumors with CK5/6 positivity identified in our study are suggestive of basoluminal subtype.

Normal-like or multiple marker negative (MMN) subtype tumors have been shown to be negative for basal markers such as CK5 and CK7 as well as negative for other molecular markers which include EGFR, c-kit, vimentin and Ki67 [13, 30]. We have found that the majority of normal-like subtype tumors express CK8/18 with absence of CK5/6 suggesting that these cells were most probably derived from luminal gland cell.

This study has also showed a significant relationship among all subtypes with p53, in keeping with a previous report [7]. Locally advanced breast cancer with *TP53* gene mutations often showed resistance to either chemotherapy or radiotherapy [36]. TP53 mutations were more frequent in basal-like and HER2+/ER- subtypes compared to luminal subtype tumors [2, 8]. Overexpression of p53 was also more frequent in cancers associated with either *BRCA1* or *BRCA2* germline mutations [37].

In our study, overexpression of CK8/18 was significantly seen in all tumor subtypes and accordingly was deemed unsuitable for specific luminal marker and tumor classification [11]. The majority of normal-like and basal-like subtypes expressed luminal keratins (CK8/18), albeit at lower levels than those found in other subtypes [12]. The expression of CK8/18 with absence of basal markers in breast cancer indicated that the tumor originated from luminal glandular cell [1]. Foulkes WD *et al.* (2003) hypothesized that *BRCA1* wild type may act as stem cell regulator and thus promote differentiation of gland epithelium in normal breast tissue [38]. As a consequent, tumors of *BRCA1* mutation expressed less CK8/18 [39].

Russo J *et al.* (2001) [40] suggested that breast cells in women with *BRCA1* mutation failed to differentiate to acinus breast cell resulting in increased risk of developing breast cancer [38]. Basal-like subtype tumors with *BRCA1* mutation were shown to be negative for ER, PR and HER2 but positive for p53 and basal markers such as CK5/6 or CK14 [7, 38, 39]. In addition, CK5/6 positive tumors were also five times (5×) more likely to be associated with *BRCA1* mutations [27].

In this study, we have also observed the morphologic features of basal-like subtype tumors, which include comedo-type necrosis, pushing invasion borders, central scar and squamous cell differentiation [12, 41].

Conclusions

Overall, all subtypes were significantly associated with race, mean size tumor, mean number of lymph nodes, histological grade and all biomarkers except EGFR and Ki67. We have found that luminal A subtype tumors are likely to be associated with low histological grade and low expression of p53. By contrast, basal-like subtype was significantly associated with Malay ethnic background, high histological grade and high expression of p53. All subtypes showed high expression of CK8/18, indicating its inappropriate role in distinguishing tumor subtypes.

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