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RELATIONSHIP BETWEEN THE SOCIO-DEMOGRAPHIC FACTORS AND THE CLINICOPATHOLOGICAL CHARACTERISTICS WITH THE INTRINSIC BIOMARKERS OF BREAST CARCINOMA OF WOMEN IN PAHANG, MALAYSIA

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ABSTRACT

Globally Breast cancer is the most common malignancy in women. The aim of this retrospective study was to determine the relationship between the socio-demographic factors and clinicopathological characteristics, with the known predictors of aggressive biological behaviour of breast cancer women in Pahang, Malaysia.The biomarkers included in this study were estrogen receptor (ER), cyclin E, p27 and nm23. Routine formalin-fixed, paraffin sections of tumor samples were used and immunohistochemically stained with antibodies against ER, cyclin E, p27 and nm23 in 93 cases of breast carcinoma in women attending at Breast clinic in Hospital Tengku Ampuan Afzan, Pahang, Malaysia during January, 1996-December, 2000. All markers studied were more common in the older age group >45 years and were roughly equally distributed among the Malays and Chinese with the exception of cyclin E which is more commonly expressed among the Malays (54.5% versus 43.9%). Breast carcinoma was found to be more common in patients over age 45 years. Most patients were diagnosed at a relatively early stage and were ER positive. ER positivity was more common in the older age, in tumors < 2cm in diameter, in early stages and in tumors with low histological grade. ER positivity was found more in patients who were alive for more than 25 months after their initial diagnosis. The cancer patients survival was significantly associated with ER (p=0.034) and nm23 (p=0.011), ER and tumor stage (p=0.031). Cyclin E expression appeared to influence the recurrence rate of primary tumor which may be a reflection of the secondary effect of treatment modality. Both markers p27 and nm23 were significantly associated with ER (p<0.001). The prognostic value of individual biological factors could be more effective in combination as opposed to single factors.

Keywords: Demographic, breast cancer, biomarkers, ***ER, cyclin E, p27, nm23,*** immunohistochemistry.

INTRODUCTION

Breast cancer is the most common malignancy among women, excluding non-melanoma skin cancers; and the second most leading cause of cancer deaths in women today lagging only behind lung cancer (Ries *et al*., 2004; Parkin *et al.,* 2002; Jokhio and Ansari, 2005). There is a strong inherited familial risk of breast cancer in some families and recent data reveals up to 27% of breast cancers may be attributed to inherited factors and mutated *BRCA1* and *BRCA2* genes are responsible for approximately 30-40% of inherited breast cancers (Lichtenstein *et al*., 2000). Some racial groups have a higher risk of developing breast cancer, notably; women of North American and European descent have been noted to have a higher rate of breast cancer than women of African and Asian origin (Parkin *et al*., 2001). Risk factors for breast cancer include, increasing age, genetics, personal and family history of breast cancer, past history of benign breast conditions, prolonged reproductive period, delayed childbirth and null parity, obesity, high fat diet, previous radiation therapy and hormonal replacement therapy beside smoking and daily consumption of alcohol (King and Schottenfeld, 1996). In Malaysia, breast cancer has been the top leading cause of cancer death among women (Yip and Ng, 1996; Joseph, 1998). The incidence of breast cancer in Malaysia is estimated to be around 27 per 100,000 populations, with close to 3,000 new cases annually. Acording to the second report of the Malaysian National Cancer Registry in 2003, breasts cancer was the most common cancer in all ethnic groups and comprised 30.4% of all newly diagnosed cancer cases among Malaysian women. The majority of women initially diagnosed with breast cancer are aged between 40 and 49 years. Breast cancer also seems to be predominant among Chinese women with an incidence of 25 per 100,000 population and Malay women with an incidence of 16 per 100,000.

What kills women with breast cancer is not the original tumor but the tumor's spread to other sites. The initial tumor can be removed by surgery, disease that has spread to nearby lymph nodes may be effectively treated by radiation therapy (Joshi *et al*., 2008) and the risk of distant metastases can be decreased by chemotherapy. However, chemotherapy is not always be an effective means of treatment and has significant side effects. Only 20 to 30 percent of women with node-negative breast

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cancer will develop metastatic disease (Schwartz *et al*., 1993). If physicians could predict who was at risk they could increase the intensity of chemotherapy for those women and reduce or even eliminate the therapy and its side effects for those not at risk.

Recently published data demonstrates that ovarian hormones, principally estrogens, play a major role in the etiology of breast cancer by affecting the rate of breast epithelial cell proliferation, perhaps via stimulation of the expression of genes encoding for growth factors (Key and Pike, 1988; Thomas *et al*., 1997; Lippman and Dickson, 1989). Intracellular ERs bind and transfer estrogen to the nucleus, where it interacts with estrogen response elements on DNA, thereby activating nearby target genes and resulting in the synthesis of proteins involved in cell division (Goodman, 1988; Brody *et al*., 1994). Although ERs exist in normal breast epithelial cells to regulate breast development during puberty and pregnancy, they are usually present in extremely low quantities (Pike *et al*., 1993; Ricketts *et al*., 1991). On the other hand, 30% of premenopausal and 60% of postmenopausal breast cancers have measurable ERs (McCarty *et al*., 1983; Huang *et al*., 2000). In general, tumors expressing these receptors tend to respond more favorably to hormonal therapies and have a better overall outcome than tumors not expressing ERs or PRs (Clark and McGuire, 1988).

The WHO estimates that at least one-third of all cancers including breast cancer are preventable (Albert, 1991) and if possible enlightened Public Health Policy should prioritize prevention and early diagnosis.

There have been an ever-expanding number of potential prognostic factors reported for patients with breast cancer. Prognostic factors correlate with survival independent of systemic therapy, and are used to select patients at risk (Wyld *et al*., 2003; Esteva and Hortobagyi, 2004; Jakic-Razumovic *et al*., 2005; Bare *et al*., 2006). The prognostic significance of histological grade has been reported by several investigators (Bay, 2006). Histological type and grade of cancer, tumor size, lymph node (Callagy *et al*., 2006), metastasis, stage and certain immunohistochemical markers like estrogen receptor (ER)/progesterone receptor (PR) status and C-erbB-2 have consistently been shown to be important prognostic factors for breast cancer survival. Poorer prognosis is associated with larger, higher grade tumors, with axillary lymph node involvement, distant metastasis, negative hormone status and positive C-erbB-2 staining (William, 1997). Unfortunately, the standard tools for assessing risk of metastasis such as the size of the tumor, its grade, the presence of estrogen receptors and the proportion of dividing cells are probably insufficient for clinical decision making (Osborn *et al*., 2004).

The expression of molecular markers in breast cancer has been examined extensively in order to provide early diagnosis and prediction outcome (Naguib *et al*., 1999; Bay, 2006; Kröger *et al*., 2006). Thus, looking for "biomarkers", proteins expressed at varying levels in early breast cancer, to provide clues about the expected behaviour of cancer and whether it is likely to spread or has spread, is a target worth aiming at. Currently, many biomarkers, particularly the hormonal and epidermal growth factor receptors, are being utilized for breast cancer prognosis. Unfortunately, none of the biomarkers in use have sufficient diagnostic, prognostic and/or predictive power across all categories and stages of breast cancer. It is recognized that more useful information can be generated if tumors are assessed with multiple markers. But choosing the right combination of biomarkers is challenging, because 1) multiple pathways are involved, 2) up to 62 genes and their protein products are potentially involved in breast cancer-related mechanisms and 3) the more markers evaluated, the more the time and cost involved (Osborn *et al*., 2004; William, 1997). It has been reported that there was significance ethnic variations in the incidence of breast cancer among women (Ahmad, 2003).

For all of these reasons, our objective of this retrospective study was therefore to examine the correlations of the markers with the socio-demographic and histo-pathological data of the breast cancer among women attending Breast clinic in Hospital Tengku Ampuan Afzan (HTAA) Kuantan, Pahang, Malaysia. In this study, we evaluated the interrelationship of the expression of the cell cycle controller gene products p27, a negative regulator, and cyclin E (Keyomarsi *et al*., 1994; Sgambato *et al*., 1997; Tan *et al*., 1997; D1Arciero *et al*., 2003; Keyomarsi *et al*., 2003; Hlupić *et al*., 2004) a positive regulator, together with the expression of the tumor suppressor gene nm23 (Hlupić *et al*., 2004; Zhao *et al*., 2004) and one of the steroid receptors, the estrogen receptor (ER).

MATERIALS AND METHODS

Patients

The study included 100 randomly collected histologically confirmed breast carcinoma cases. The cases were all women who attended the breast clinic in Hospital Tengku Ampuan Afzan (HTAA), Kuantan, Pahang, Malaysia, during January, 1996- December, 2000. The criterion for inclusion was determined by the availability of sufficient tissue for H & E (Hematoxylin and eosin) staining and further immunohistochemical studies. Based on theses criteria 93 cases were included in the present study. The available demographic and clinicopathological data were collected from the biopsy request forms or patient's files wherever possible. However, for some patients we couldn't obtain sufficient relevant information due to patient default, incomplete entry of data in patient's files or their referral to another center for treatment. At least

two blocks of the tumor tissue from each patient were analyzed to reconfirm the original histological diagnosis and to determine the histological grade. Tumors were classified in accordance with NHSBSP guidelines and histological grading was established using modified Bloom's grading system described by Elston and Ellis (1991). The tumors were staged according to the American Joint Committee on Cancer staging system, grouping patients based on the tumor size (T), lymph node status (N), and distant metastases (M) into 4 stages, thus allowing clinicians to derive prognostic information necessary for therapeutic decisions (Fleming *et al*., 1997).

Immunohistochemistry

Formalin-fixed paraffin sections of breast cancer tissue were mounted on glass slides coated with 3-aminopropyl-triethoxysilane (APES; Sigma, Poole, Dorset, UK) and were baked for 30 min at 56–60°C, before being dewaxed in xylene. The tissue sections were rehydrated by sequential immersion in 100% and 50% ethanol to distilled water and then subjected to heat antigen retrieval for 40 min in citrate buffer (pH 6) in a jar containing preheated (95-99 ^{0}C) target retrieval solution. After cooling, tissue sections were incubated for 5 min in 0.3% (v/v) hydrogen peroxide. Subsequently, the sections were washed in tap water and Tris-Buffer (pH 7.45) and were exposed to normal rabbit serum (diluted with Tris) for 30 min at room temperature (20–24°C). Diluted primary antibody (anti-ER) was applied and incubated overnight at 4°C (18 hours). After washing with Tris, biotinylated rabbit anti-goat secondary antibody, together with the Strept-AB Complex/HRP (0377, DAKO, Glostrup, Denmark) was applied for 30 min at room temperature. Staining was revealed by development in the chromogen 3, 3-diaminobenzidine tetrahydrochloride (DAB) for 5–30 min. Sections were rinsed with distilled water and were mounted using cover slip with aqueous bases mounting medium.

Evaluation of immunohistochemistry

The intensity of immunostaining was labeled as +, less than 25% of cells stained intensely, ++, 25-50% of cells stained intensely, +++, 51-75% of cells stained intensely and ++++, more than 75% of cells stained intensely. The pattern of staining varied (nuclear, cytoplasmic, membranous) depending on the type of antibody used e.g. against ER receptor, cyclin E, p27 and nm23. A single pathologist scored all immunohistochemistry and the binary system (positive versus negative) was used for the out come of all of the markers.

STATISTICAL ANALYSIS

The data were analyzed for significant associations between the expression of immunohistochemical tumor markers and other tumor biological characteristics and behavior by the non-parametric Chi square method using SPSS 11.0 for windows. Differences were considered significant when $p<0.05$.

RESULTS

In the period studied, from January, 1996 to December, 2000, 93 confirmed cases of breast carcinoma were included in this investigation. Table 1 shows age distribution in investigated breast carcinoma patients. The mean age of the patients included in the study was 51.3 ranging between 32 and 89 year old. The majority of patients (60%) were 41-60 years of age. Table 2 summarizes the distribution of all investigational parameters (clinicopathological characteristics) in 93 breast carcinoma cases included in this study. The results of the quantitative immunohistochemical assays were correlated with clinical and histological findings such as patient age, ethnic distribution, breast side, histological type, tumor size, lymph node metastatis, distant metastatis, stage and tumour grade. The ethnic distribution was comparable in Malays and Chinese constituting 50% and 44% of the total cases respectively. The Indian constituted only 6.5%. The most frequent histological type of breast cancer (93.6%:87/93 cases) in this study was infiltrating ductal carcinoma. The others were two non-invasive ductal carcinoma, two papillary carcinoma and one each medullary and mucinous carcinoma. Most of the patients (57%) had tumor size ranging between 2-5 cm in diameter (T2) and lymph node metastasis was observed in 50.5% of cases while distant metastases was observed in only 2 cases at the time of diagnosis. At the time of presentation 71% of the patients were in stage 2 and 60% had histological grade 2 tumors. Most of the patients (64.5%) presented with right sided tumors.

Table 1. Age distribution in investigated breast carcinoma cases (93).

Age group (years)	Number of cases (percentage)
31-40	15 (16.1%)
41-50	38 (40.9%)
51-60	18 (19.3%)
61-70	16 (17.2%)
71-80	05 (5.4%)
81-90	01 (1.1%)

Information about parity of the patents were available for only 44 cases, 19 of these were nulliparous, 14 had 1-3 children and rest of 11 had more than 3 children. Family history of cancer was available for 45 cases, 4 cases had family history of breast cancer, 3 cancers other than breasts and rest of 38 had no family history of any cancerous lesion. Out of 43 cases with information about prior hormonal treatment only seven had history of hormonal treatment in past few years. Eight cases were unmarried out of 44 for which marital status was known.

Table 2. Distribution of all investigational parameters (clinicopathological characteristics) in 93 breast carcinoma cases.

Factors	*Cases (%)
Age	
< 45	30 (32.3)
> 45	63 (67.7)
Ethnic distribution	
Malays	46 (49.5)
Chinese	41 (44.1)
Indians	06 (6.5)
Breast side affected	
Right	60 (64.5)
Left	33 (35.5)
Histological type of cancer	
Infiltrating ductal carcinoma	87 (93.5)
Non-infiltrating ductal carcinoma	02 (2.2)
Others	04 (4.3)
Tumor size	
T1	14 (15.1)
T2	53 (57)
T3	18 (19.4)
T4	08 (8.6)
Lymph node metastatis	
N0	46 (49.5)
N1	44 (47.3)
N2	03 (3.2)
Distant Metastasis	
M0	91 (97.8)
M1	02 (2.2)
Stage	
I	11 (11.8)
II	66 (71)
III	14 (15.1)
IV	02 (2.2)
Histological grade of tumor	
I	19 (20.4)
II	56 (60.2)
III	18 (19.4)

* Number of patients for which data were available.

The intrinsic biomarkers included in this study were the steroid receptor for estrogen (ER), the oncogene and tumour suppressor gene products such as cyclin E, p27 and nm23. The intensity and extent of staining in tumor tissue is graded as mentioned in material and methods. The overall presence of the cell cycle controller gene products cyclin E, p27, the anti-metastasis protein nm23 and estrogen receptor (ER) are shown in table 3. The nm23 shows the lowest expression 41.9% and ER the highest 54.8%. Cyclin E and p27 were expressed in 47.3% and 52.7% of breast cancers respectively. Most of the breasts cancer tissues (54.8%) were found positive for ER. Nuclear staining was positive in all cases, however 38 cases revealed variable degree of cytoplasmic staining too. The tumors with positive marker expression showed mostly mild to moderate staining intensity with variable degree of nuclear and cytoplasmic staining (Figs. 1-4). The association of the tumor markers for ER, cyclin E, p27, and nm23 overexpression with other classical prognostic factors is shown in table 4. All markers were more commonly expressed in tumors of the older age group > 45 years than in those ≤ 45 (29.1%-38.7% vs. 11.8%-20.4%).

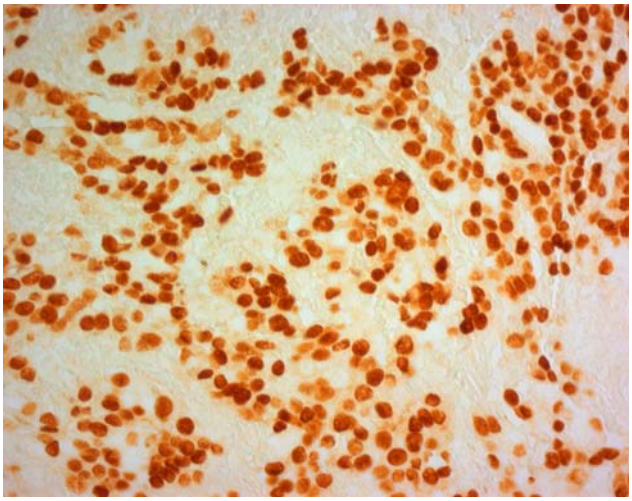

Fig. 1. Immunohistochemical staining of estrogen receptor (ER) in an infiltrating ductal carcinoma, which is entirely localized to the nuclei of the tumor cells (40 HPF).

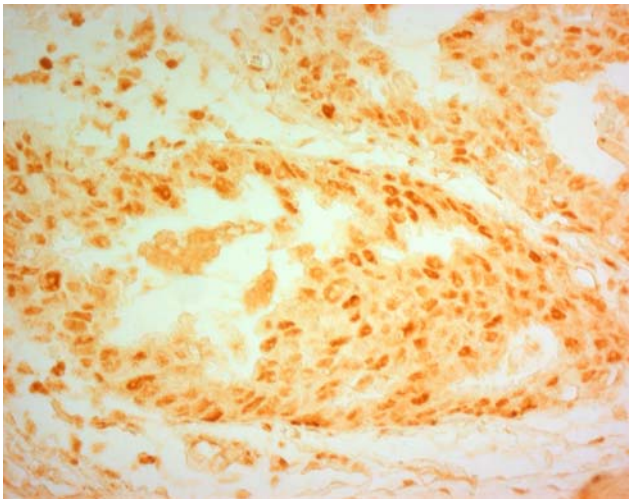

Fig. 2. Immunohistochemical staining of cyclin E in an infiltrating ductal carcinoma, which varies in intensity but still localized to the nuclei of tumor cells (40 HPF).

The ethnic distribution revealed approximately equal presence of all the markers among the Malays and the Chinese except for the cyclin E which showed substantially higher positivity rate among the Malays (54.5% vs. 43.9%), however, the difference is not quite

statistically significant. Since most tumors involved the right breast (64.5%), all markers were consequently more often expressed on that side but even when positivity was calculated within the breast side still marker expression for cyclin E (50.8% vs. 39.4) and ER (57.6 vs. 51.5) was higher in right-sided tumors. nm23 showed the least expression in infiltrating ductal carcinomas as compared to the other markers (39.8% vs. ≥ 45.2), however, none of the markers showed significant association with a particular histological type probably because of the very small number of the non-infiltrating ductal carcinoma and the other types of cancer. However, nm23 showed the lowest positivity rate in non-metastasizing tumors when compared to other markers (39.8% vs. ≥ 46.2).

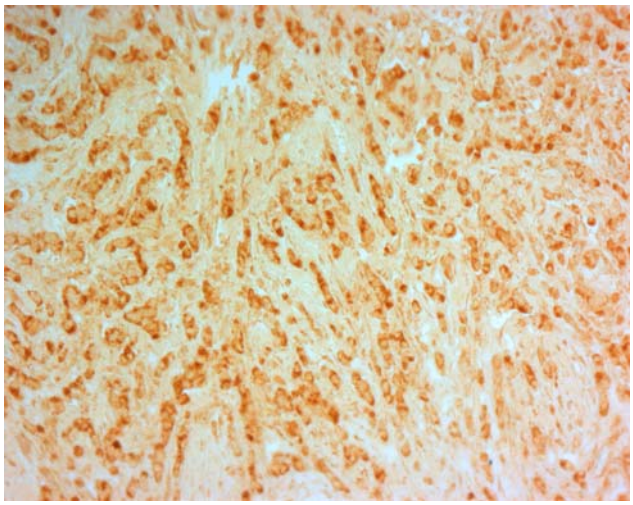

Fig. 3. Immunohistochemical staining of p27 in an infiltrating ductal carcinoma, which varies in intensity and is not only localized to the nuclei of the tumor cells (40 HPF).

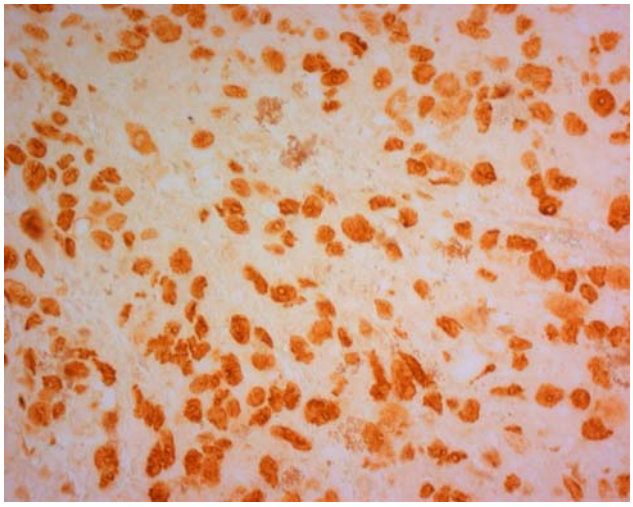

Fig. 4. Immunohistochemical staining of nm23 in an infiltrating ductal carcinoma, which is mostly confined to the nuclei of the tumor cells (40 HPF).

Table 3. Frequency and staining intensity of tumor biomarkers ER, cyclin E, p27 and nm23 in 93 breast carcinoma cases.

Tumor biomarkers	Staining Intensity	Cases* (%)
ER	Negative	42 (45.2)
	+	18 (19.4)
	++	17 (18.3)
	+++	11 (11.8)
	++++	5 (5.4)
Cyclin E	Negative	49 (52.7)
	+	17 (18.3)
	++	16 (17.2)
	+++	8 (8.6)
	++++	3 (3.2)
P27	Negative	44 (47.3)
	+	10 (10.8)
	++	20 (21.5)
	+++	11 (11.8)
	++++	8 (8.6)
nm23	Negative	55 (59.1)
	+	9 (9.7)
	++	18 (19.4)
	+++	6 (6.5)
	++++	5 (5.4)

* Number of patients for which data were available.

ER positivity was more common in patient over 45 year of age rather than in patients below 45(37/63 vs. 14/30). Both non-infiltrating ductal carcinoma and 3 of the 4 other types were positive for ER. Most of the tumors of less than 2 cm size (T1), (12/14) were positive for ER. Tumors with or without axillary lymph node metastasis had similar rate for ER positivity. 43 of 77 low stages (1 & 2) were positive for ER compared to 8 of 16 high stage tumors. More of low histological grade (G1) tumors (13/19: 68.4%) stained with ER antibody than the high histological grade (G2 & G3) (38/74: 51.4%). As for histological grade and stage only ER positivity showed significant association with earlier stages of cancer.

As shown in table 5, most of the patients alive without recurrence were variably positive for all different markers. Longer survival period was significantly associated with nm23 ($p = 0.011$) and ER ($p = 0.034$) positivity (Table 6). 25 of 39 cases with localized disease and 3 of 7 dead cases were ER positive. Most of the cases with 25-48 months survival period (19/25: 76%) and those with more than 48 months survival (12/21:57.1%) were positive for ER.

No significant association could be found between tumor size and markers expression. At the same time no substantial differences could be seen in between markers regarding their expression status in tumors of varying

Table 4. Crosstabulation of the association of the tumor markers for ER, cyclin E, p27, and nm23 over expression with other prognostic factors in 93 breast carcinoma cases.

Characteristics	Cyclin E		p27		nm23		ER	
	Positive~ cases (%)	Negative^ cases (%)	positive~ cases (%)	negative^ cases (%)	positive~ cases (%)	negative^ cases (%)	Positive~ cases (%)	negative^ cases (%)
Age								
≤ 45 Y	14 (15.1)	16 (17.2)	17 (18.2)	13 (14)	11 (11.8)	19 (20.4)	14(15.1)	16 (17.2)
> 45 Y	30 (32.2)	33 (35.5)	32 (34.4)	32 (34.4)	27 (29.1)	36 (38.7)	37 (39.8)	26 (27.9)
Ethnic distribution								
Malays	25 (54.5)	21(45.7)	24 (52.2)	22 (47.8)	21 (45.7)	25 (54.2)	26 (27.9)	20 (21.5)
Chinese	18 (43.9)	23 (56.1)	21 (51.2)	20 (48.8)	17 (41.5)	24 (58.5)	23 (24.7)	18 (19.4)
Indians	1 (16.7)	5 (83.3)	4 (66.7)	2 (33.3)	0	6 (100)	02 (2.2)	04 (4.3)
Breast side								
Right	30 (32.6)	29 (31.5)	32 (34.8)	27 (29.3)	25 (27.2)	34 (37)	34 (36.6)	26 (28)
Left	13 (14.1)	20 21.7)	49 (53.3)	16 (17.4)	13 (14.1)	20 (21.7)	17 (18.2)	16 (17.2)
Histological type								
IDC*	42 (45.2)	44 (47.3)	47 (50.5)	39 (41.9)	37 (39.8)	49 (52.7)	46 (49.5)	41(44.1)
NIDC[#]	1 (1.1)	1 (1.1)	0	2 (2.2)	1 (1.1)	1 (1.1)	02 (2.2)	0
Others	1 (1.1)	4 (4.3)	2 (2.2)	3 (3.2)	0	5 (5.4)	03 (3.2)	01 (1.1)
Tumor size					5 (5.4)	9 (9.6)	12 (12.9)	02 (2.2)
T1	4 (4.3)	10 (10.8)	9 (9.7)	5 (5.4)				
T2	26 (28.0)	27 (29.0)	28 (30.1)	25 (26.9)	20 (21.5)	33 (35.5)	26 (27.9)	27 (29)
T3 & T4	14 (15.0)	12 (12.9)	12 (12.9)	14 (15.1)	13 (14.0)	13 (14.0)	13 (14)	13 (14)
Lymph node metastatis								
N0	19 (20.4)	27 (29.0)	22 (23.7)	24 (25.8)	14 (15.1)	32 (34.4)	25 (26.9)	1 (22.6)
N1 & N2	25 (26.9)	22 (23.7)	27 (29.0)	20 (21.5)	24 (25.9)	23 (24.8)	26 (27.9)	21 (22.6)
Distant Metastasis								
M0	43 (46.2)	48 (51.6)	47 (50.5)	44 (47.3)	37 (39.8)	54 (58.1)	49 (52.7)	42 (45.1)
M1	1 (1.1)	1 (1.1)	2 (2.2)	0 (0.0)	1 (1.1)	1 (1.1)	02 (2.2)	0
Stage								
I & II	35 (37.6)	42 (45.2)	42 (45.2)	35 (37.7)	30 (32.3)	47 (51.6)	43 (46.2)	34 (36.4)
III & IV	9 (9.7)	7 (7.6)	7 (7.6)	9 (9.7)	8 (8.6)	8 (8.6)	08 (8.6)	08 (8.6)
Histological grade								
I	7 (7.5)	12 (12.9)	10 (10.8)	9 (9.7)	6 (6.5)	13 (14.0)	13 (14)	06 (6.5)
II & III	37 (39.8)	37 (39.8)	39 (41.9)	35 (37.7)	32 (34.4)	42 (45.1)	38 (40.9)	36 (38.7)

Number of positive (~) and negative (^) cases with data, *Infiltrating ductal carcinoma, Non-infiltrating ductal carcinoma.

Table 5. Association of intrinsic tumor markers status with clinical outcome of 93 breast carcinoma cases.

Clinical outcome	Cyclin E		P27		Nm23		ER	
	+ve cases (%)	-ve cases (%)	+ve cases (%)	-ve cases (%)	+ve cases (%)	-ve cases (%)	+ve cases (%)	-ve cases (%)
Alive without recurrence	16 (17.2)	11 (11.8)	15 (16.1)	12 (12.9)	10 (10.8)	17 (18.3)	15 (16.1)	12 (12.9)
Alive with recurrence	9 (9.7)	3 (3.2)	9 (9.7)	3 (3.2)	7 (7.5)	5 (5.4)	10 (10.8)	02 (2..2)
Dead	1 (1.1)	6 (6.5)	2 (2.2)	5 (5.4)	3 (3.2)	4 (4.3)	03 (3.2)	04 (4.3)
Lost to follow up	18 (19.4)	29 (31.2)	23 (24.7)	24 (25.8)	18 (19.4)	29 (31.2)	23 (24.7)	24 (25.8)
P value	0.02		0.223		0.611		0.171	

sizes. Lack of significant association was also noted between markers expression and lymph node involvement or distant metastasis. Only ER positivity showed significant association with earlier stages (I & II) of cancer than late stages (III & IV). Significant association between tumor clinicopathological features and intrinsic tumor markers was observed for tumor stage and patient survival with ER and for clinical outcome with cyclin E (Table 7*)*. Tumor markers p27 and nm23 expressed significant association ($p = <0.01$) with ER but no significant association was found between the other markers (Table 8).

Table 6. Association of intrinsic tumor markers status with the survival of 93 breast carcinoma cases from HTAA Kuantan during 1996-2001.

Patients' survival in months	Cylin E		P27		Nm23		ER	
	+ve cases (%)	-ve cases (%)	+ve cases (%)	-ve cases (%)	+ve cases (%)	-ve cases (%)	+ve cases (%)	-ve cases (%)
<12	6 (6.5)	5 (5.4)	7 (7.5)	4 (4.3)	3 (3.2)	8 (8.6)	6 (6.5)	5 (5.4)
12-24	9 (9.7))	6 (6.5)	9 (9.7)	6 (6.5))	9 (9.7)	6 (6.5)	8 (8.6)	7 (7.5)
25-48	10 (10.8))	15 (16.1)	15 (16.1)	10 (10.8)	15 (16.1)	10 (10.8)	19 (20.4))	6 (6.5)
>48	12 (12.9)	9 (9.7)	11 (11.8)	10 (10.8)	8 (8.6))	13 (14)	12 (12.9)	9 (9.7)
Not determined	7 (7.5)	14 (15.1)	7 (7.5)	14 (15.1)	3 (3.2)	18 (19.4)	6 (6.5))	15 (16.1)
P value	0.380		0.337		0.011		0.034	

Table 7. Association between tumor clinicopathological features and intrinsic tumor markers (p values for chi^2 tests)

Characteristic	Tumor Markers							
	ER		Cyclin E		P27		Nm23	
	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
Patient alive <1Y, 1-2Y, 3-4Y, >4Y	0.034		0.380		0.337		0.011	
Tumor size	0.069		0.419		0.736		0.737	
Clinical outcome	0.171		0.020		0.223		0.611	
Metastasis	0.194		0.939		0.175		0.790	
Axillary lymph node metastasis	0.274		0.460		0.203		0.110	
Histological grade	0.327		0.592		0.722		0.595	
Tumor stage	0.031		0.502		0.318		0.827	
Treatment	0.142		0.034		0.060		0.162	

Table 8. Association in between tumor markers ER, cyclin E, P27 and nm23 (p values for chi^2 tests)

Prognostic Markers	Cyclin E	p27	nm23
ER	0.231	0.001	0.009
Cyclin E	-	0.045	0.993
P27	-	-	0.208

DISCUSSION

Breast cancer is a complex disease that still imposes a significant healthcare burden on women worldwide. The hunt for prognostic markers of human cancers in general and breast cancers in particular continues to haunt investigators who keep struggling to elucidate the right ones that serve as the best prognostic and therapeutic predictors to be used in routine clinical practice (Pedrini *et al*., 2004). Currently, many biomarkers, particularly the hormonal and epidermal growth factor receptors, are being utilized for breast cancer prognosis (Pedrini *et al*., 2004). Unfortunately, none of the biomarkers in use have sufficient diagnostic, prognostic and/or predictive power across all categories and stages of breast cancer. It is recognized that more useful information can be generated if tumors are evaluated with multiple markers (William, 1997). Breast cancer risk increases markedly after 40 year of age. A women's chance of breast cancer increases from one out of 235 at age 40 to one in 54 at age 50 and then it continues to increase further with increasing with age (Ferlay *et al*., 2001). The age standardized incidence rate for breast cancer is 54.9 per 100,000 women per year in Singapore (Bay *et al*., 2006). In the present study 78 of 93 cases were in age group above 40. The Second report of Malaysian National Cancer Registry shows that most of breast cancers occurred in age group 40-60 (Lim and Yahaya, 2003) which is comparable to our results as 57/93 cases (61.3%) were in the same age group in our study.

Ethnic distribution of breast cancer was almost same as that reported in Malaysian National Cancer Registry report (Lim and Yahaya, 2003) and both Malay and Chinese patients had comparable distribution with slight predominance of the former, however, Indians were underrepresented in our study and constituted only 6.5% of total cases.

The predominant histological type of breast carcinoma in this study was infiltrating ductal carcinoma as reported in most of other studies (Fleming *et al*., 1997). Most of the patients (72%) presented with tumor size < 5 cm in diameter (T1-T2), while 46 of 93 cases (49.5%) had no metastasis to lymph nodes and only two cases were confirmed to have distant metastatic spread. These

findings suggest that most of the patients with breast cancer in our series presented at an early stage of the disease where 77 of 93 cases (82.8%) were either in Stage 1 or Stage 2. This indicates the efficacy of the breast cancer awareness programs carried out by the NGOs and Malaysian government agencies (Mahathevan, 2002).

In our study we described the individual status four well known immunohistochemical markers cyclin E, p27, nm23 and ER in the breast cancer tissues, and attempted at measuring the association of each with the various demographic and clinicopathological criteria of the cancer patients and also with each of the other markers. Although the higher cyclin E positivity rate among Malays as compared to Chinese in Malaysia (Table 4) is not statistically significant, it might still reflect genuine genetic difference between the tumors in the two races but failed the statistical test due to the small number of cases recruited in this study.

Histologically, most of the breast cancers were either in Grade 1 (20.4%) or Grade 2 (60.2%), once again indicating relatively good prognosis. Previous studies have confirmed the histological grade association with prognosis and chemotherapeutic response (Elston and Ellis, 1991; Pinder *et al*., 1998).

Interestingly most of cases (64.5%) had right sided breast cancer in contrary to various other reports where left sided cancer was the commonest (Senie *et al*., 1980). It has been suggested that in about 55% of women, the left breast is slightly larger than the right and therefore contains a larger amount of tissue at risk for becoming cancerous (Senie *et al*., 1980). It will be interesting to evaluate this hypothesis regarding the increased incidence of right sided breast cancer in our patients. However, this discrepancy may be due to the limited number of cases in the present study.

Most of the patients were treated with only surgery (49 of 93 cases), most probably due to their presentation in early stages. The rests of the cases were additionally treated with other adjuvant modalities such as chemotherapy and radiation. Survival data and follow up of patients was not maintained according to the standard practice, as 50% of cases were lost to follow up after a few visits to hospital. However, 46 patients had follow up data available for more than 25 months and 21 of them for more than 48 months after their first diagnosis. Only 7 cases were confirmed to be dead in this series by July 2004 (the last date of follow up). Although longer follow up periods are required (at least 5 years), our data collectively suggest a better prognosis in this series of patients which we believe is due to the earlier stage at the time of presentation and also probably due to the appropriate treatment these patients received.

Thirty three of 44 cases (75%) with breast cancer were either nulliparous or had 1-3 children which indicate that reduced fertility is one of the common associations with breast cancer as shown in previous studies (King and Schottenfeld, 1996). Only 4 out of 45 had family history of breast cancer which is in confirmation with the previous data of familial breast cancer representing 5-10% of total breast cancers (King and Schottenfeld, 1996; Douglas, 2002). However, only 7 of 43 cases had prior history of any hormonal treatment in our study.

Moreover, patients with ER positive tumors have prolonged disease free survival after primary treatment, superior overall survival and longer survival after recurrence compared with patients having ER-negative tumors, which is independent of axillary node status (McGuire *et al*., 1990). 54.8% (51/93) of the tumors in this study were positive for ER especially those with tumor size less than 2 cm in diameter (T1). Moreover, ER positivity was significantly more in stage 1 and 2 compared to stage 3 and 4 ($p<0.05$). Most of the cases having follow up for 25-48 months or more than 48 months were also ER positive and only 3 of 7 patients who died during our study period had ER positive tumor. This clearly indicates the good prognosis of tumors with positive ER status.

The significant association of early stage breast cancer and prolonged survival with ER positivity in our patients indicating that breast carcinomas expressing estrogen receptors have better prognosis because they are more differentiated and favorably respond to hormonal treatment compared with ER negative tumors (Clark and McGuire, 1988; McGuire *et al*., 1990). Moreover, patients with ER positive tumors have prolonged disease free survival after primary treatment, superior overall survival and longer survival after recurrence compared with patients having ER-negative tumors (McGuire *et al*., 1990).

The better survival ($p=0.011$) of those with increased expression of nm23 is probably related to the anti-metastatic effect of this marker (Zhao *et al*., 2004), however no significant association could be established between nm23 and lymph node and distant metastasis (Table 4). Marginal association though a non-significant one was noted between decreased nm23 expression and lymph node metastasis ($p = 0.11$).

The influence of cyclin E on clinical outcome ($p=0.02$) is probably attributed to its influence on treatment strategy and its association with other poor prognostic parameters like high grade, late stage and negative estrogen receptor status (Donnellan and Chetty, 1999). However, it could not be substantiated in our present study.

The two markers p27 and nm23 that are known to have anti-tumor effects (Osborn *et al*., 2004; William, 1997; Tan *et al*., 1997; Porter *et al*., 1997) and thus favorably influence prognosis were unsurprisingly found to be significantly associated with ER which also has a favorable prognostic influence (Hlupić *et al*., 2004).

CONCLUSION

The present study showed that most of the breast cancer patients attending the breast clinic in HTAA Kuantan, present at an early stage and are ER-positive, thus having good prospect for favorable prognosis if appropriately treated. *nm23* in this study appears to be the only marker besides ER that significantly influenced patients' survival while cyclin E had an influence on clinical outcome. The combination of markers examined in this study might have an influence on the prospective biological behavior of breast tumors and the ultimate survival and well being of these patients. The prognostic value of individual biological factors could, therefore, be more effective in combination as opposed to single factors (Schindlbeck *et al*., 2005). Since we are still a long way, short of accurately defining the right combination of prognostic markers. It is suggested, that a more comprehensive and statistically reliable data can be obtained, if a much larger number of breast cancer cases from all over Malaysia is to be included in this type of research work.

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