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ORIGINAL ARTICLE

An analysis of predictive biomarkers in routine histopathological reporting of infiltrating ductal breast carcinoma in a tertiary hospital in Malaysia with a focus on limitations and directions for future development

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Abstract

Predictive biomarkers such as oestrogen (ER) and progesterone (PR) receptors and c-erbB-2 oncoprotein have become a staple in breast cancer reports in the country as they increasingly play an important role in the treatment and prognosis of women with breast cancers. This study reviews the practice of histopathology reporting of these biomarkers in a Malaysian tertiary hospital setting. Retrospective data on demographic, pathological and biomarker profiles of patients with invasive ductal carcinoma who had undergone mastectomy or lumpectomy with axillary node clearance from 2005 to 2006 were retrieved from the Department of Pathology, Penang Hospital and analysed. The prevalence of ER positivity (55.8%), PR positivity (52.5%), c-erbB-2 oncoprotein overexpression (24%) and triple negativity (ER negative, PR negative, c-erbB-2 negative) (15%) by immunohistochemistry were comparable with other studies. Notably, c-erbB-2 overexpression was equivocal (2+) in 15% of cases. Since about a quarter of equivocal (2+) cases usually show amplification by FISH, a small but certain percentage of patients would miss the benefit of anti-c-erbB-2 antibody therapy if FISH is not performed. New ASCO/CAP guidelines on the quantitation of ER and PR will probably increase the prevalence of ER/PR positivity, invariably leading to significant ramifications on the management of patients as more patients would be deemed eligible for endocrine therapy, as well as categorisation of triple negative breast cancers.

Key words: breast cancer, oestrogen receptor, progesterone receptor, c-erbB-2 oncoprotein

INTRODUCTION

Breast cancer is one of the most common cancers in women in most parts of the world, accounting for approximately 22% of all female cancers. The prevalence rises to about 26% in more affluent countries, thereby making breast cancer twice as common when compared to cancers of other organs in women.[1] In Malaysia, the incidence of breast cancer is reported to be 39.5 per 100,000 population, and this accounts for 31% of all newly diagnosed female cancers.[2] Breast cancer is the most common cancer in women in Penang, accounting for 35.5% of all female cancers. Up to 74.4 % of these breast cancers are histologically of the infiltrating ductal type.[3] The role of histopathology in the provision of prognostic and therapeutic information for breast cancer has been well established for many years. Prognostic factors are defined as clinical, biological or pathological factors which influence the outcome of the patient when, or before the cancer is manipulated by systemic therapy. Over the years, a wide range of factors as well as experimental markers have been identified, investigated and reported in various literature with regards to clinical relevance as prognostic markers in breast cancer patients. These prognostic factors can be broadly categorised into two groups - traditional factors and biomarkers. Traditional factors are those that can be assessed during conventional examination and histological evaluation of

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tumours. Biomarkers are less readily available in some laboratories, but some, such as hormone receptor expression, have become important in predicting therapeutic response.

Since the introduction of some predictive biomarkers (i.e. hormone receptor expression, c-erbB-2 oncoprotein overexpression) in the histopathological reporting of breast cancers, these markers have progressed to become a staple in breast cancer reports in the country as they increasingly play an important role in the treatment and prognosis of women with breast cancers.

In view of the significance of breast cancer in this country and the importance of these biomarkers, this study aims to review the practice of histopathology reporting of these predictive biomarkers in a Malaysian tertiary hospital setting, as exemplified by Penang Hospital, in order to appreciate the limitations of this practice, and directions for improvement and future development.

MATERIALS AND METHODS

Selection of cases

A total of 120 patients who were diagnosed with an infiltrating ductal carcinoma of the breast, NOS, and had subsequently undergone a mastectomy or lumpectomy with axillary lymph node dissection between 1 January 2005 and 31 December 2006 at the Penang Hospital, were included in this study. Demographic and pathological information were retrieved retrospectively from request forms and histopathological reports archived in the Department of Pathology, Penang Hospital. Pathological parameters retrieved included the largest gross dimension of the tumour (in mm.), histological grade (modified Bloom and Richardson) and lymph node status (presence or absence of metastatic deposits). This study excluded all patients diagnosed with an infiltrating ductal carcinoma but did not subsequently undergo a mastectomy or lumpectomy with axillary lymph node clearance for whatever reason. Invasive breast carcinomas of other histological types were also excluded.

The haematoxylin and eosin (H&E) and immunohistochemical slides of the cases included in this study were traced from the pathology archives and reviewed, using an Olympus binocular light microscope (model CX31), for biomarker expression.

Immunohistochemistry

Oestrogen (clone 1D5) and progesterone (clone PgR) receptor status were determined via standard immunohistochemistry on archived formalin-fixed, paraffin-embedded tissue in all of the cases (1:50 dilution, EnVision, DAKO Cytomation). Quantitation of a "positive" hormone receptor result was set at a minimum of 10% nuclear staining of invasive tumour cells (Figure 1).

c-erbB-2 oncoprotein expression status was also determined via standard immunohistochemistry performed on archived formalin-fixed, paraffin-embedded tissue of the cases (1:100 dilution, Code-NR A0485, DAKO Cytomation). Staining of only invasive cancer cells were evaluated; intraductal components were not accepted in the evaluation. A "positive" result was considered when there was membrane immunostaining; cytoplasmic staining was disregarded. Only tumour cells showing a score of 3+ were considered as exhibiting c-erbB-2 oncoprotein overexpression. Tumour cells with membrane staining of similar intensity to the internal control (non-neoplastic breast epithelium) were considered as not overexpressed. A score of 0 was given if there was no staining observed, or if there was membrane staining of less than 10% of the tumour cells. A score of 1+ (not overexpressed) was given if there was a faint/barely perceptible membrane staining detected in

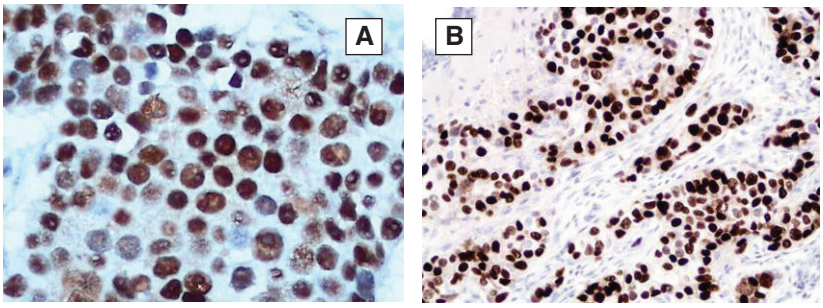

FIG. 1: (A) Oestrogen (X 400) and (B) progesterone (X 100) receptor protein positive nuclear staining. IHC.

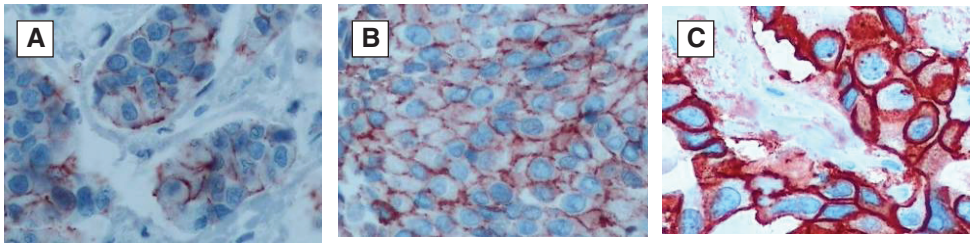

FIG 2: c-erbB-2 oncoprotein staining expressions. (A) $^{1+}$, (B) $^{2+}$ and (C) $^{3+}$. IHC X 400.

more than 10% of the tumour cells. These cells also showed only partial membrane staining. A score of 2+ (equivocal overexpression) was given for a weak to moderate complete membrane staining observed in more than 10% of the tumour cells. Finally, a score of 3+ (overexpressed) was given if there was a strong, complete membrane staining observed in more than 10% of the tumour cells (Figure 2).

Statistical analysis

All data collected were entered into a data management system and analysed using SPSS for Windows (version 11.0). Significance of correlations was set at a p value of <0.05.

RESULTS

Demographic profile of patients

The mean (56 years) and median (57 years) ages of the patients were fairly similar while the mode was 52 years. The ages of these patients ranged from 31 years to 87 years. The most frequent age-group was 51 to 60 years of age. The majority of patients (70.8%) were above 50 years of age while only 5.8% were 40 years of age and below.

More than half the cases (68 patients) were of Chinese ethnicity whilst the second largest racial group were the Malays (36 patients). Indians contributed 12.5% of the cases whilst one patient was of Eurasian heritage.

Pathological profile

The recorded maximum dimensions of tumours ranged from 10 mm to 115 mm (mean = 39 mm; mode = 30 mm). Almost 87% of the patients had either a Grade 2 or Grade 3 tumour. Only 13% were Grade 1 tumours. Nodal metastases were seen in 67.5% of patients.

Predictive biomarker profile

Oestrogen (ER) and progesterone (PR) receptor positivity was observed in 55.8% and 52.5% of the tumours respectively. c-erbB-2 oncoprotein was overexpressed (3+) in 24% (Table 1). 18 (15%) cases were "triple-negative" (negative expression of ER, PR and c-erbB-2 oncoprotein).

c-erbB-2 oncoprotein overexpression showed a statistically significant association with histological grade ($p = 0.011$); higher grade tumours had a higher prevalence of c-erbB-2 oncoprotein overexpression (Spearman's rank correlation coefficient test $r = 0.264$, $p = 0.004$). A converse relationship was identified between oestrogen ($r = -0.321$, $p = 0.002$) and progesterone receptor status ($r = -0.282$, $p < 0.001$) with c-erbB-2 oncoprotein overexpression.

TABLE 1: Comparison between c-erbB-2 overexpression with ER and PR expression by immunohistochemistry in infiltrating ductal carcinoma, Penang Hospital

c-erbB-2 expression	ER+/PR+ No. (%)	ER+/PR- No. (%)	ER-/PR+ No. (%)	ER-/PR- No. (%)	Total No. (%)
0	32 (26.7)	5 (4.2)	3 (2.5)	18 (15)	58 (48.3)
1+	9 (7.5)	1 (0.8)	3 (2.5)	2 (1.7)	15 (12.5)
2+	9 (7.5)	2 (1.7)	1 (0.8)	6 (5)	18 (15)
3+	6 (5)	3 (2.5)	1 (0.8)	19 (15.8)	29 (24.2)
Total	56 (46.7)	11 (9.2)	8 (6.6)	45 (37.5)	120 (100)

A significant association was identified between oestrogen and progesterone receptor status ($p < 0.001$). Oestrogen receptor status also showed a statistically significant ($p = 0.021$) association with age and tumour size. A higher prevalence of oestrogen receptor positivity were noted in older women ($r = 0.233$, $p = 0.010$ Spearman's rank correlation coefficient test) and smaller tumours [Chi-Square test; $p = 0.014$; Spearman's rank correlation coefficient test ($r = - 0.261$, $p = 0.004$)].

No similar statistically significant associations (Fisher's exact test) were found between progesterone receptor status with age and tumour size ($p = 0.189$).

Higher grade tumours were noted to have a higher prevalence of oestrogen receptor negativity [Chi-Square test; $p = < 0.001$; Spearman's rank correlation coefficient test ($r = -0.409$, $p < 0.001$)] and progesterone receptor negativity [Chi-Square test; $p = < 0.001$; Spearman's rank correlation coefficient test ($r = -0.425$, $p < 0.001$)].

There were no statistically significant associations between nodal metastases and oestrogen receptor ($p = 0.498$), progesterone receptor ($p = 0.704$) and c-erbB-2 oncoprotein overexpression ($p = 0.074$) [after Yates' Correction for Continuity (which compensates for the overestimation of chi-square values when using a 2x2 table)].

DISCUSSION

The demographic (age and ethnicity) and pathological (tumour size, grade and nodal involvement) profiles in this study showed similarities as well as slight differences from other studies held both within and outside of Malaysia. The predictive biomarker profiles of the tumours were however comparable with other studies.

Demographic profile

The median age-group of this study was 57 years, which was slightly older but almost comparable with a previous study done in Penang Hospital in which the median age group was 54 years.[4] This figure was however marginally younger than the median age in most developed countries. Only 29% of our patients were under 50 years of age, differing from previous studies in Kuala Lumpur and Penang where the proportion of cases below 50 years of age have been reported to be 40 to 50%.[4,5,6] Possible contributions to the difference are that our study have excluded cases that did not undergo mastectomy/lumpectomy with axillary node clearance, and also, being from a public hospital, it would not have captured the cohort of younger, more affluent women who may have preferred to utilise the private hospitals in Penang.

In our study, almost 57% of the patients were Chinese and 30% were Malays. The ethnic distribution of female admissions for Penang Hospital during the study period was 47% Chinese and 30% Malay. Based on this, it might appear that the incidence of breast cancer is probably higher in ethnic Chinese. A higher incidence of breast cancer in Chinese has also been reported in the Second Report of the National Cancer Registry and the Penang Cancer registry.[3]

Pathological profile

The mean tumour size in our study was 38.8 mm. This is higher than mean tumour sizes reported from the United States (mean = 24.3)[7] and Hong Kong (mean = 23.5 mm).[8] As with most studies, tumour size exhibited a statistically significant association with nodal metastases ($p = 0.003$). A weak but statistically significant positive correlation was observed between tumour size and number of lymph node metastases ($r = 0.252$, $p = 0.006$). This finding is comparable to most studies which showed that tumour size was one of the most important prognostic factors in breast cancer. Many large studies have shown that patients with smaller tumours have a significantly better survival and lower incidence of nodal metastases. The incidence of nodal metastases in the literature[9] varies slightly between different studies but they all tended to show an increase in incidence of nodal metastases as tumour size increases. Our study suggests that our patients were presenting for management at a later stage than more advanced countries.

87% of tumours in our study were either of Grade 2 or Grade 3, findings which were comparable to a study conducted at the University of Malaya Medical Centre in which 82% of tumours were either Grade 2 or Grade 3 tumours.[10]

Predictive biomarkers

c-erbB-2 overexpression (3+) was identified in 24% of the cases whilst a further 15% were equivocal (2+). The 3+ expression prevalence compares well with most published accounts of c-erbB-2 oncoprotein overexpression, which range from 15 to 30%.[11,12]

HER2/c-erbB-2 is an oncogene that encodes for a cell surface receptor protein in the epidermal growth factor family. This protein, a member of the subfamily of structurally related type I tyrosine kinase receptors, is an important regulator of cell growth, differentiation and survival. Gene amplification and protein overexpression have been used to select patients who may respond to anti-c-erbB-2 therapy and have also been linked to poorer outcome, especially in node positive patients.[13] Treatment with anti-c-erbB-2 antibody has been shown to improve survival when used alone or in combination with chemotherapy in patients with metastatic breast cancer. More importantly, newer data now show that c-erbB-2 overexpression can be used as a predictor for primary treatment response. For example, some data have shown that c-erbB-2 overexpression may correlate with enhanced response to anthracyclines.[14] Other studies have also shown that c-erbB-2 overexpression may confer resistance to adjuvant treatment with cyclophosphamide/methotrexate[15] and tamoxifen.[16]

In view of the significance of c-erbB-2 oncoprotein overexpression in the management of breast cancer, it is therefore important for the pathologist to provide an accurate determination of c-erbB-2 expression status on every case. c-erbB-2 status in most laboratories in Malaysia are predominantly attained via immunohistochemical assays. Fluorescence in-situ hybridisation (FISH) or silver enhanced in-situ hybridisation (which is still awaiting US Food and Drug Administration [FDA] approval) for detection of c-erbB-2 amplification has only been recently introduced in the last few years and is available in few centres in Malaysia. The need for FISH analysis can be seen in this study where c-erbB-2 overexpression (3+) was identified in only 24% of the cases whilst a further 15% of cases were equivocal (2+). Studies have shown that about a quarter of equivocal (2+) cases usually show amplification by FISH.[9] Therefore, if anti-c-erbB-2 antibody treatment is based solely on immunohistochemistry, a small but certain percentage of patients would miss the significant benefit of the therapy.

Even though most literature regarding immunohistochemical testing of c-erbB-2 suggest that the 2+ score is the most problematic, some authors also believe that the misinterpretation of 3+ scores is also of importance. Even though the overwhelming majority of 3+ cases exhibit gene amplification by FISH, Mass *et al* has shown that 11% of these 3+ tumours actually do not exhibit any evidence of gene amplification.[17] In view of the costs involved in performing FISH, some centres have opted to perform FISH only on equivocal (2+) cases and have omitted 0, 1+ and 3+ cases. False negative results i.e. immunohistochemistry negative c-erbB-2 and FISH positive cancers are rare in the 0 to 1+ scores. It is important to consider the cost versus risk of only performing FISH on 2+ cases as it is usually the false positive 3+ cases not confirmed by FISH that are the most harmful, both in terms of cost [as anti-c-erbB-2 antibody treatment is expensive] and the deleterious side effects to the patient (i.e. risk of cardiotoxicity). Studies have also shown that anti-c-erbB-2 antibody treatment may not be beneficial to the patient if no c-erbB-2 amplification is detected as treatment correlates better with gene amplification.

The American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) have recently modified the criteria for 3+ by increasing the percentage of positive cancer cells from 10% to 30%, in an attempt to reduce a small percentage of false 3+ positive cases caused by intense staining owing to edge artifacts. With this modified criteria, cases with 11% to 30% cancer cell positivity would be classified under 2+ positivity and would therefore need FISH for confirmation.

In an ideal situation, confirmation of c-erbB-2 gene amplification via FISH for all 2+ and 3+ cases by immunohistochemistry (and not only 2+ cases) ought to be considered due to the price and morbidity associated with anti-c-erbB-2 antibody therapy as well as the risk of false positivity for c-erbB-2 oncoprotein overexpression. It makes little sense to try and save on the costs of a FISH test, when a one-year course of anti-c-erbB-2 antibody treatment can cost more than RM100,000.

This study also incidentally showed that c-erbB-2 oncoprotein overexpression had a weak negative correlation with oestrogen ($r = -0.321$, $p = 0.002$) and progesterone receptor status ($r = -0.282$, $p < 0.001$), which may explain the relative resistance of c-erbB-2 positive cases towards selective endocrine modulators such as tamoxifen. c-erbB-2 oncoprotein overexpression also has a statistically significant association with histological grade of tumour ($p = 0.011$).

In our study, 56% of the cancers were ER positive and 52% were PR positive. 45 % were positive for both receptors whilst 16.7 % were positive for either one (6.7% were ER

negative/PR positive and 10% were ER positive/ PR negative). The percentage of ER receptor expression was comparable with the University of Malaya Medical Centre study which reported a 58.4% rate of ER expression from 2004 to 2008.[10]

ER and PR status have been widely studied and positive ER/PR expression has been found to be a weak favourable prognostic factor. Its main significance lies in the selection of patients for endocrine (anti-oestrogenic) therapy. A "positive" result was set at a minimum of 10% nuclear staining, which was the recommended practice at the time of the study. This was based on findings by several studies which found that using 10% nuclear staining as the basis for a positive result was reproducible and correlated well with clinical response to endocrine (anti-oestrogenic) therapy.[18] The American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) have however recently released guidelines recommending a 1% or more cut-off value for both ER and PR receptor status, as levels as low as 1% positive staining of cancer cells are also associated with significant clinical response to endocrine therapy.

Using the "old" cut-off value of 10% nuclear staining, 62.5% of our patients would have been eligible for endocrine therapy as women with either ER or PR positive tumours would have been treated since studies have also shown a relationship between PR positivity and disease outcome and endocrine therapy response.[19] Based on the new ASCO/CAP guidelines, the percentage of ER and PR positivity should increase from previously reported values. This would invariably lead to significant ramifications on the management and treatment of patients as more patients would be eligible for endocrine therapy.

In this study (as with some laboratories in Malaysia), hormone receptor status was reported as either "positive" or "negative". Some studies have however shown that quantitation of hormone receptor status using immunohistochemistry correlates closely with biochemical results and can be used as a prognostic factor. Studies by Harvey and co-workers in 1999 using the Allred score have demonstrated a linear correlation between the Allred score and ER content, as well as differences in disease free survival between different scores.[20] Data from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B14 clinical trials suggested that the benefit of tamoxifen was limited to patients with higher levels of hormone receptor (ER) expression.[12] Therefore, it is now recommended that some form of semi-quantitation be performed on all hormone receptor positive cases. The problem lies in the standardisation of the semi-quantitation method to be used in the laboratories in Malaysia (i.e Allred score, H – score, Q score etc.) so that the data obtained can be of use between various institutions. Further studies also should be conducted to determine the benefits of endocrine therapy in patients with lower levels of hormone receptor expression.

This study identified a statistically significant association between ER and PR status ($p < 0.001$), a finding that is not surprising as progesterone receptor expression is believed to be a test complementary to ER that increases the power of ER. It is thought that false positive ER (ER positive/PR negative) results may indicate the existence of functionally inactive variants of ER whilst false negative results (ER negative/ PR positive) may be a result of splice variants of ER that are not detected by the assays but are functional. In this study, 37.5% of cases were negative for both receptors.

15% of cases in this study were strict "triple negative" breast cancers (i.e. tumours with negative expression for ER, PR and c-erbB-2 oncoprotein). While studies have shown an increased likelihood of distant recurrences and death in women with "triple negative" breast cancers,[21] it is less clear whether ER and PR negative cancers with 1+ c-erbB-2 expression have an equally poor prognosis. The definition of "triple negative" defers between studies, with some defining such tumours as having negative expression of ER, PR and c-erbB-2/ HER2, whereas others define them as ER and PR negative tumours without overexpression of c-erbB-2/HER2. To further confound the situation, the criteria for ER and PR negativity also varies depending on whether a cut-off of 10% or 1% is used.[22, 23, 24]

It was also interesting that this study showed a statistically significant association ($p = 0.016$) and a weak negative correlation between tumour size and oestrogen receptor status ($r = - 0.261$, $p = 0.004$), a feature that has also been noted in another study.[25] Unfortunately, no scientific evidence backed explanation is available to explain this occurrence.

This study also showed a statistically significant association between oestrogen ($p = < 0.001$) and progesterone ($p = < 0.001$) receptor

status with histological grade of tumour. Both oestrogen receptor and progesterone receptor status showed a moderate negative correlation with histological grade, meaning that higher grade tumours tended to be more often hormone receptor negative. This finding is consistent with what has been reported so far in the literature where tumour types that tend to be ER positive include tubular, mucinous, papillary and ductal carcinomas of good (low) histological grade.[26]

ER status also showed a statistically significant association with age. When analysed further, a weak positive correlation ($r = 0.233$, $p = 0.010$) was found between age and oestrogen receptor positivity, a finding noted in some texts.[27] The University of Malaya Medical Centre study had also reported lower ER positive rates for women younger than 40 years of age.[10] Unlike ER however, the progesterone receptor (PR) positive rate is more likely to be higher in younger or premenopausal women, probably as a result of greater oestrogen stimulation. However, in this study, no statistically significant association with age was identified.

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