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ARTICLE

Significance of p53, Bcl-2, and HER-2/*neu* Protein Expression in Omani Arab Females with Breast Cancer

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Racial disparity in the presentation of breast cancer and the outcome of its treatment is well established. However, the causes remain unexplained. The scarcity of reports about the prognostic significance of p53, bcl-2, and HER-2/*neu* in Arab females with breast cancer has been the impetus to this study. We evaluated the prognostic significance of altered expression of p53, bcl-2, HER-2/*neu* in Omani Arab females with non-metastatic breast cancer with correlation to other established prognostic factors. We have retrospectively analyzed the immunohistochemical expression of p53, HER-2/*neu* and bcl-2 in paraffin embedded blocks of 72 females diagnosed with invasive breast cancer between 1992 and 2002. The expression of the above proteins was correlated with other prognostic factors and univariate and multivariate analysis was carried out for all prognostic factors. Overexpression of p53 significantly correlated with younger age (<40), pre-menopausal status, poor differentiation with inverse correlation with bcl-2 expression. Expression of bcl-2 immunopostivity significantly correlated to low histological grade and positive estrogen and progesterone receptor status. On univariate and multivariate p53 overexpression and lack of bcl-2 immunostaining resulted in worse survival outcome, but not Her-2/*neu* overexpression. Expression patterns of p53 and bcl-2 are independent predictors of survival in Omani Arab population which may help to stratify these patients into different risk groups. (Pathology Oncology Research Vol 9, No 4, 226–231)

Keywords: breast cancer, p53, bcl-2, HER-2, arab-females

Introduction

Breast cancer is a heterogeneous disease with variable biological and clinical characteristics. The racial influence in invasive breast cancer in terms of age at presentation, clinico-pathological features, and outcome of treatment has been widely reported.[1-3] It has been established that breast cancer in many Asian and African countries tends to affect younger females, presents in advanced stage with poor prognostic features, and has worse outcome when compared to their counterparts in the Western countries.[4-9] There is no doubt that the lack of early detection program and awareness contribute to advanced presentation, however, the biological aggressiveness in terms of poor differentiation, lack of steroid receptor expression, and tendency to affect younger females remain unexplained. Abnormalities described in the structure and activity of several proto-oncogenes may contribute to the development or progression of breast cancer.[10] Alterations in the proto-oncogenes p53, bcl-2, and HER-2/*neu* have been extensively studied in Western and East Asian females with breast cancer and proposed as prognostic markers of potential clinical utility.[11-21] However, few studies exist on prognostic significance of these oncogenes in female breast cancer in developing countries particularly from Arab world.[22,23] To our knowledge, this is the first correlative study from the Arab countries of the Persian Gulf that investigated the role of these biomarkers in the progression and prognosis of breast ductal neoplasia in Arab females in Oman. We analyzed the expression levels of p53, bcl-2, and HER-2/*neu* proteins by immunohistochemistry and evaluated their relationship to other established prognostic factors and their prognostic significance.

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Materials and Methods

Breast Cancer Specimens

Paraffin-embedded breast tumor blocks were obtained from 72 women with breast cancer who were diagnosed at the Sultan Qaboos University Hospital in Muscat, Oman, between 1992 and 2002. The clinical information regarding age, sex, tumor type, lymph node status, stage, and therapy, overall survival was obtained for analysis. All the tumors were infiltrating ductal carcinomas.

All specimens were histopathologically typed according to the World Health Organization classification and histologically graded according to the modified Scarff–Bloom-Richardson histological grading. All specimens were evaluated without knowledge of the clinical data.

Immunohistochemical staining

For bcl-2 and p53 immunostaining, 5 m thick sections were obtained from formalin-fixed, paraffin-embedded tumor tissues. Tissue sections were deparaffinized in xylene and rehydrated through graded alcohols. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide in water for 5 minutes, the sections were placed in 1600 ml of antigen masking solution (Vector Laboratories, Birmingham. Ca94010) in boiling pressure cooker for 5 minutes for p53, and Progesterone receptor PR immunostaining or placed in 500 ml of antigen masking solution heated in a microwave oven at 750 W for 20 minutes for bcl-2, and estrogen receptor (ER) immunostaining. Following antigen retrieval, the sections were incubated with the following antibodies: monoclonal antibody to bcl-2 protein (clone 124, Dako, synthetic peptide sequence comprising amino acids 41–54 of bcl-2 protein) at dilution of 1:50 and monoclonal antibody to p53 protein (DO7, Dako, amino acid residues 19–26 of human p53, reacts with both mutant and wild-type human p53 protein) at dilution of 1:40 for 30 minutes, monoclonal antibody for ER (clone 105, Dako) at dilution of 1:40, and monoclonal antibody for PR (clone PgR 636, Dako) at dilution of 1:50. The antigens were detected by incubation with Envision system peroxidase (Dako) for 30 minutes. Peroxidase was demonstrated with 0.5% diaminobenzidine (DAB; Sigma) for 10 minutes and the sections were counterstained with Mayer's hematoxylin. Positive and negative controls were performed at same time for each section. Sections from normal lymph node and colonic adenocarcinoma served as positive control for bcl-2 and p53 immunostaining respectively. Negative controls were obtained by incubation of parallel slides omitting the primary antibodies. The bcl-2 staining was considered as positive if more than 30% of the tumor cells showed cytoplasmic immunoreactivity and the p53 staining was interpreted as positive when >10% of the tumor cells showed distinct nuclear staining.[12,24]

Similar sections were also stained for HER-2/*neu* according to instructions of the manufacturer and using reagents included in HerceptTest Kit. The primary antibody in the kit was polyclonal in pre-diluted form. A known case of invasive ductal carcinoma was used as positive control. A negative control was used where primary antibody was omitted. Following the criteria recommended by Dako for the HercepTest Kit, Overexpression of HER-2/*neu* was defined as membranous staining in more than 10% of the neoplastic cells. Partial or incomplete, weak to moderate, and moderate to strong membranous staining in more than 10% of the tumor cells were scored as 1 (negative), 2 (weak positive) and 3 (strong positive), respectively. The consensus was viewed as conclusive for indeterminate cases.

Statistical analysis

Associations between p53, bcl-2, and HER-2/*neu* immunostaining and tumor clinic-pathological features including histological grade, tumor size, lymph node involvement and ER/PR status, were assessed using the chi-squared test. Survival time was measured from diagnosis to death censored by the end of follow-up. Actuarial curves for overall survival (OS) were obtained and graphs constructed using the Kaplan–Meier method. Univariate analysis was performed using Kaplan-Meier-product limit estimates of survival distribution, and differences between survival curves were tested using the log-rank test for the following variables: age, menopausal status, tumor differentiation, size, lymph node status, ER/PR status, p53, bcl-2, and HER-2/*neu* immunostaining. The influence of all variables considered in univariate analysis on overall survival was estimated using the multivariate Cox's proportional hazard models. The statistical analysis was carried out using *SigmaStat SigmaPlot*® statistical analysis software.

Results

Clinical and Pathological Characteristics

Patient and tumor characteristics are listed in *Table 1*. The mean age of all patients was 49.6 years (range from 28 to 80 years) and 29 patients (40.3%) were pre-menopausal. All tumors included were invasive ductal carcinoma and 33 (46%) had a tumor larger than 5 cm in diameter. There were 4 patients in histological grade I, 39 in grade 2 and 29 in grade 3. Lymph node metastasis was noted in 32 (44.4%) patients. The overall staging for the 72 patients was stage I (5 patients), stage II (31 patients), and stage III (34 patients).

Sixty-four patients had surgery with modified radical mastectomy and lumpectomy performed in 51 and 21 patients, respectively. A total of 53 patients (73.6%) received chemotherapy, 13 patients and 40 patients in neo-adjuvant and adjuvant setting respectively. Thirty-eight

patients received various protocols of anthracycline-based regimens and 15 patients received CMF regimen. Radiotherapy was administered in 35 patients (48.6%) and adjuvant hormonal treatment was given to 48 patients (66.7%).

Frequencies and associations p53, bcl-2 and c-erbB-2 expression

p53, bcl-2, and HER-2/*neu* oncogene overexpression was detected in 30 (41.7%), 39 (54.2%), and 13 (19.4%) of cases, respectively (*Figure 1,2,3*). Expression of p53 immunopositivity significantly correlated to lack of estrogen and progesterone receptors expression (p=0.009) and (p=0.015), and poor tumor differentiation (p=0.0001). There was a strong inverse correlation between p53 and bcl-2 expression (p=0.000). p53 overexpression tended to occur in younger than 40 years and pre-menopausal patients (p=0.02 and 0.015 respectively). There was no correlation with size of tumor, lymph node involvement, or HER-2/*neu* expression. Expression of bcl-2 immunopostivity significantly correlated to low histological grade (p=0.000), and positive PR and ER status (p=0.000) (Table 1). No association was found between HER-2/*neu* immunopostivity and patient age, tumor grade, estrogen and progesterone receptor expression, lymph node involvement, tumor size, or p53 and bcl-2 receptor expression.

Table 1. **Patients' characteristics and relationships of p53, bcl-2 and HER-2/*neu* overexpression to clinicopathological features and hormone receptor**

	Positive p53		*Positive bcl-2*		*Positive c-erbB-2*	
Variable	No	P	No.	P	No.	P
Whole Series	30	39	13			
Age						
<40	17	0.02	31	0.34	1	0.2
>40	13		8		12	
Menstrual status						
Pre-menopause	18	0.015	16	0.9	3	0.27
Post-menopause	12		23		10	
Histologic grade						
G1/G2	9	0.0001	31	0.000	7	0.87
G3	21		8		6	
Size						
<5cm	14	0.66	24	0.11	7	0.9
>5cm	16		15		6	
Lymph node						
Absent	18	0.3	19	0.3	8	0.8
Present	12		20		5	
ER						
Negative	19	0.009	98	0.000	7	0.8
Positive	11		31		6	
PR						
Negative	21	0.015	11	0.000	8	0.8
Positive	9		28		5	
p53						
Negative			29	0.000	8	0.96
Positive			5		5	
Bcl-2						
Negative	22	0.0001			5	0.94
Positive	8				8	
HER-2/neu						
Negative	27	0.9	31	0.8		
Positive	5		8			

Survival Analysis

The follow-up period ranged from 5.9 to 112.77 months (median, 38 months). At the time of analysis, 20 (27.8%) patients had died, and 52 (72.2%) were alive. The median survival for the whole group was 59.4 months (95% CI 26.6-92.3). The overall 5-year survival rate was 50%. In Univariate analysis, bcl-2 and p53 immunoreactivity were strongly inversely associated with survival. The median survival was 80.1 months (95% CI 41-119.2), for p53 negative cases but 36 months (95% CI 17.49-54.5), for p53 positive patients (P=0.001) (*Figure 4*). The median survival was 112.8 months for bcl-2 positive cases but only 42.03 months for bcl-2 negative patients (P=0.003, 0.004) (*Figure 5*). The other significant prognostic variables in the univariate analysis were histologic grade (p=0.001), and size (> 5cm) (p=0.02), and age less than 40 years (p=0.05). In multivariate analysis, performed by introducing all variables in the Cox model, p53 overexpreesion (HR: 4.5 95% CI 1.1-18.5; P=0.04), lack of bcl-2 expression (HR: 3.7 95% CI 1.4-28.2; P=0.03), size (> 5 cm) (HR: 7.48 95% CI 1.7-32.5; P=0.007) and lymph node involvement (HR: 5.24 95% CI 1.6-17.4; P=0.007) retained prognostic significance.

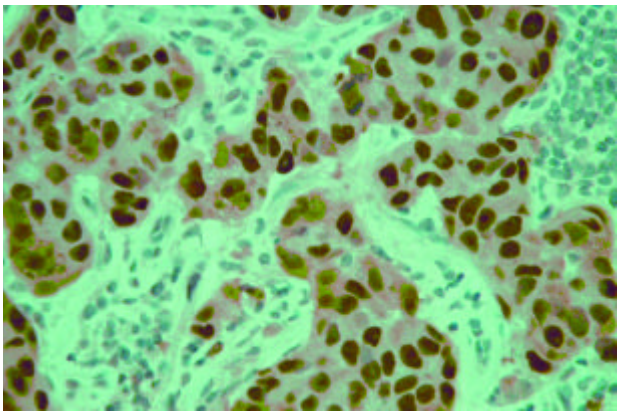

Figure 1. *Strong nuclear staining for p53 protein*

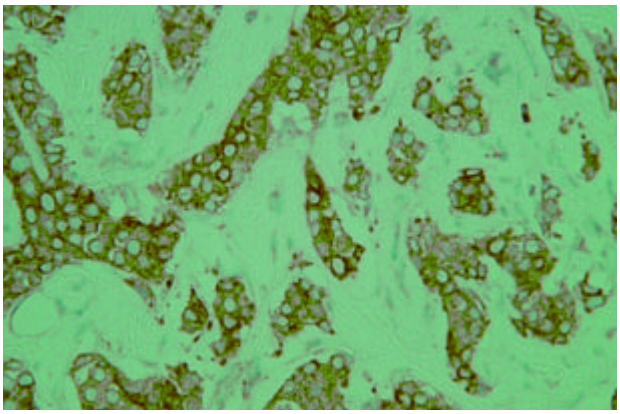

Figure 2. *Strong membrane staining for bcl-2 protein*

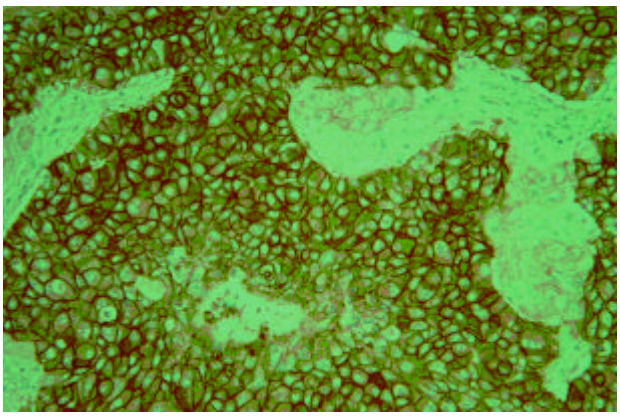

Figure 3. *Strong membrane staining for HER-2/neu protein*

Discussion

The Sultanate of Oman is a developing Asian Arabic country where breast cancer is the most common tumor in females. It accounts for one of every five cancers detected in females with age standardized incidence rate of 15.6 per 100,000 Omani females.[25] Although there is no early breast cancer detection program, the current treatment modalities are employed in a large proportion of patients who present with advanced disease. The altered expression of the oncogenic proteins has been associated with biological aggressiveness and outcome of treatment. Few studies exist on the roles of these in oncogenes in female breast cancer in developing countries particularly from Arab world and their prognostic significance.[22,23] This study examines the prognostic significance p53, bcl-2, and HER-2/*neu* oncogenes in invasive breast cancer in Oman.

Abnormalities of the *p53* gene and protein expression, which are usually associated with an allelic loss on chromosome 17, have been widely reported in breast carcinoma with variable mutation rate ranging between 40-80%.[11,26] Significant association between *p53* alterations and tumor size, histologic and nuclear grade, DNA ploidy, mitotic rate, proliferation index, positive node status, distant metastases, and lack of estrogen receptors were reported in white European and American, Afro-American, and East Asian females.[1,14,27] In our study, p53 overexpression was found in 41.7% of the tumors examined with statistically significant correlations between p53 immunopositivity and lack of estrogen and progesterone receptors expression, poor tumor differentiation, and a strong inverse correlation with bcl-2 expression (Table1). A similar p53 overexpression rate of 57.3% and correlation pattern were found by Temmim et al on their study of Kuwaiti females younger than 30 years. However, in that study p53 immunostaining was not correlated with bcl-2 and HER-2/*neu* expression or survival.[23]

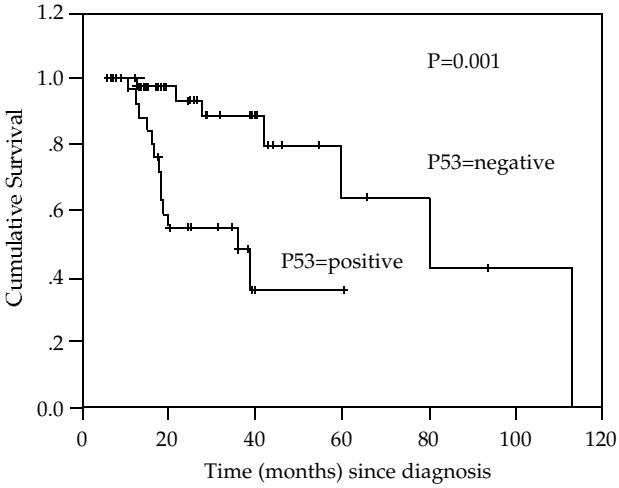

Figure 4. *Kaplan-Meier curves categorized according to expression of p53 protein in females with invasive breast cancer*

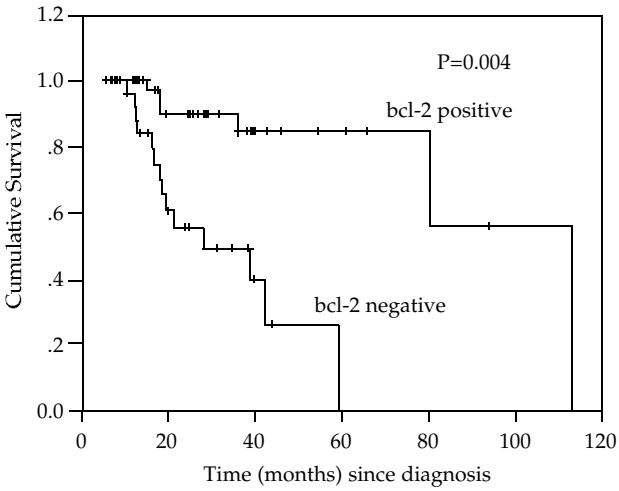

Figure 5. *Kaplan-Meier curves categorized according to expression of bcl-2 protein in females with invasive breast cancer*

Moreover, p53 expression was associated with a shorter survival in the univariate analysis (Figure 4), and was found to be an independent prognostic factor on multivariate analysis. This is consistent with report by el-Ahmady et al where altered p53 expression was shown to be of prognostic significance in 94 Arab Egyptian females.[22] Similarly, many other studies worldwide have shown that p53 overexpression is associated with early disease recurrence and poor survival,[11,12,27] but few failed to observe such associations.[28]

Several previous studies have demonstrated that bcl-2 expression is associated with favorable prognostic feature such as smaller tumor size, well-differentiated tumors, steroid receptor expression, less nodal involvement.[29-31] Expression of bcl-2 immunopostivity was found in 56.7% of the tumors which is consistent with previous reports from the West.[29,30] The expression of bcl-2 was significantly more frequent in tumors with low histological grade and positive estrogen and progesterone receptor status (*Table 1*). In 76% of cases, there was an inverse relationship between p53 and bcl-2 immunoreactivity which has been previously demonstrated.[12,24] This inverse relationship between p53 and bcl-2 has been studied in vitro where overexpression of a mutant p53 in MCF-7 induced down-regulation of bcl-2 both at protein and mRNA level.[32]

Our results suggest bcl-2 expression to be an independent prognostic factor of favorable clinical outcome. Contrary to our results, El-Ahmady found that bcl-2 is not predictive of survival in 94 Arab Egyptian females.[22] It remains unclear why bcl-2 would as an anti-apoptotic protein confers a favorable prognostic outcome. Several explanations have been put forward. For example, it has been demonstrated that the expression of bcl-2 suppresses the proliferation of developing leukemia cells[33] by a cell cycle-inhibitory function, suggesting that overexpression of the bcl-2 gene might delay the growth of cancer cells. Moreover, it has been suggested the bcl-2 protein overexpression may have a pro-apoptotic function in some circumstances by increasing the half-life of Bax protein.[34]

Patients with strong c-erbB-2 expression seem to have a poor response to hormonal agents such as tamoxifen and also to non-anthracycline chemotherapy.[35] HER-2/*neu* oncogene overexpression (+3) was found in 19% of patients, however there is no statistically significant correlation with other prognostic factors. Similarly, HER-2/*neu* overexpression was not found to be a predictor of clinical outcome (*Figure 6*). This may to due to the small number of tumors that were positive in this cohort. The vast majority of our patients received anthracycline based chemotherapy, which may explain the lack of prognostic significance of HER-2/*neu* expression.

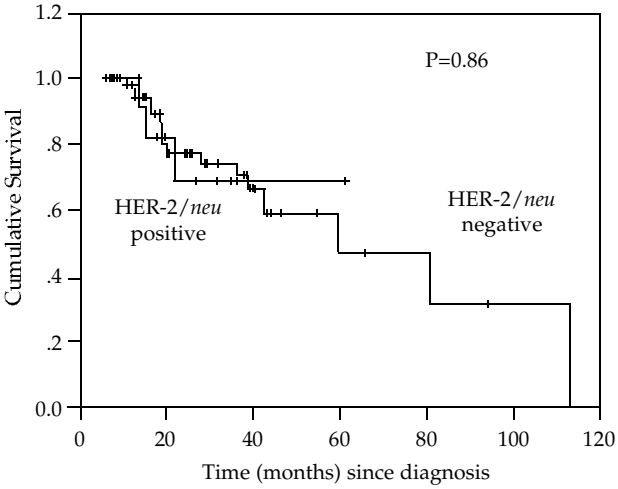

Figure 6. *Kaplan-Meier curves categorized according to expression of HER-2/neu protein in females with invasive breast cancer*

In conclusion, we have described the clinicopathological variables and immunohistochemical expression of p53, bcl-2 and HER-2/*neu* in Arab females with non-metastatic breast cancer in Oman. This subgroup of breast cancer patients had more lymph node involvement or large tumors in general. The results suggest that bcl-2 protein is associated with favorable clinicopathological features and a significant prognostic value. Overexpressions of p53 is a strong prognostic factor for overall survival independent of other clinical factors such as tumor size, histological grade and lymph nodal status.

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