Multivariate Analysis of Bcl-2, Apoptosis, P53, And Her-2/Neu In Breast Cancer: A Short Term Follow Up

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Abstract:
Background. Several molecular genetic alterations in breast cancer, including aneuploidy, altered apoptosis, aberrant expression of p53, HER-2/neu and Bcl-2, have been associated with poor prognosis in breast cancer patients. To determine the importance of molecular-genetic factors relative to more traditional surgical-pathologic prognostic factors, multivariate analysis was performed. Materials and Methods. Ninety-four fresh tissue samples of primary breast carcinoma were studied with flow cytometry for DNA ploidy. On the same specimens steroid hormone receptors (ER and PR) were measured in the cytosol fraction using Abbott ELISA assays, HER-2/neu was determined in the membrane fraction and mutant p53 protein in the nuclear fraction, both, by Oncogene Science ELIZA procedures. Bcl-2 and apoptosis (Cell death) were measured in cell lysates by Oncogene Science & Boehringer Mannheim ELISA assays. In addition, information regarding surgical-pathological features of the tumor was obtained. Multivariate analysis using unconditional logistic regression model was done to identify variables predictive of poor prognosis. Results. Using univariate analysis, histological grade, tumor stage, lymph node status, HER-2/neu, and mutant p53, were predictive of poor short-term prognosis. By multivariate analysis, tumor stage, lymph node status, HER-2/neu were independent factors. Grade subgroup analysis versus time of relapse, illustrated a predictive value of Bcl-2 in only low-grade tumors while apoptosis was significant in high-grade type. Conclusion. Among a panel of molecular-genetic factors investigated, HER-2/neu was the most strongly predictive of poor short-term prognosis in breast cancer. Patients with HER-2/neu positive tumors can benefit from Herceptin therapy.
Introduction

Breast cancer is one of the common cancers and is a leading cause of cancer mortality in women (1). In Egypt it accounts for 25% of all malignancies in women (2). The biological factors contributing to clinical aggressiveness of breast cancer can provide a basis for identification of low and high risk groups as well as help to predict tumor response to therapy (3).

A variety of prognostic factors have been evaluated to determine if it is possible to identify high-risk breast cancer patients. These factors included tumor size, nodal status (4), histological-grade (5) steroid hormone receptor status (6), DNA ploidy (7), proliferative index (8) cathepsin D (9) and analysis of growth factor receptors such as the epidermal growth factor receptor (10).

Apoptosis is a physiological process that is crucial to the growth and development of multi-cellular organisms. Its dysregulation has been linked to tumorigenesis (11). Also, several proteins have been identified to be components of the complex apoptosis machinery such as Bcl-2, which was the first protein identified to possess antiapoptotic properties (12). In addition, Bcl-2 gene expression is thought to be related to endocrine sensitivity in hormone responsive tissues including uterus (13), normal and cancerous breast tissue (14). Bcl-2 gene expression may, therefore, be of potential importance in the modulation of responsiveness to hormonal treatment in breast cancer (15).

A few previous studies have examined p53 protein expression in human mammary carcinoma utilizing immunohistochemistry showing p53 protein expression to be present in 27-54% of primary breast carcinomas (16). Wild type p53 induces apoptosis (17). Mutant p53, however, can inhibit apoptosis and increases the function of Bcl-2 (18). The proto-oncogene neu (c-erbB2, or HER-2/neu), encodes for a 185 kilodalton membrane protein related to protein kinase family (19), is overexpressed with or without gene amplification in 20-30% of breast cancers. In breast cancer patients, neu amplification or overexpression correlates with poor prognosis and tumor resistance to chemotherapy (20). In addition, the expression of oncogene neu in the mammary gland resulted in accelerated lobulo-alveolar development and formation of focal mammary tumors after a long latent period (21). Although several investigators have studied the role of HER-2/neu in neoplasia to determine if its overexpression is involved in the evolution of cells with malignant potential (22), the precise significance of the relationship between HER-2/neu overexpression and the biologic behavior in breast cancer remains
unknown.
The purpose of the current study was to simultaneously evaluate the relative prognostic weight of multiple molecular biological markers in the breast cancer patients, and compared with conventional clinicopathologic markers by performing a multivariate analysis.

**Patients and methods**
The tissue samples used in this study were obtained from 94 patients who had been admitted to the Department of surgery at Ain Shams University Hospitals in Cairo. No initial chemotherapy or hormonal therapy was performed prior to tumor excision. Surgically removed tissues were divided into three aliquots, the first was formalin fixed and paraffin embedded for histopathological diagnosis, the second was freshly processed for flow cytometric DNA analysis and the third was frozen at -80°C for performing assays for ER, PR, apoptosis (Cell death), Bcl-2, Mutant p53, and HER-2/neu, proteins.

Patients included in this study were surgically treated by modified radical mastectomy (75%) or conservative mastectomy (25%), with axillary lymph node clearance. Adjuvant post operative radiotherapy was given for those patients with conservative surgery in the form of tangential field arrangement for breast tissue and direct fields for peripheral lymphatics using either CO-60 or linear accelerator 5Mv. A booster dose was given to the tumor bed using electron beam with optimum tumor dose up to 6500cGy. Meanwhile for those patients with modified radical mastectomy, (when indicated), chest wall and peripheral lymphatics radiation was the ideal technique, with a dose 4500 cGy to the chest wall, 5000 cGy for the peripheral lymphatics. Patients with positive lymph nodes and high risk node negative group received adjuvant chemotherapy in the form of anthracyclin containing regimens (FAC) for 6 courses every three weeks Those with cardiac problems, received CMF (methotexate, fluorouracil and cytoxan). Adjuvant hormonal therapy was given for pre and post-menopausal patients with ER+ or PR + or both. Patients were followed-up to 37 months by clinical examination, laboratory investigation as well as radiological investigation.

**Chemicals and Reagents**. Nitrocellulose (NC) membranes were obtained from Bio-Rad, Hercules, CA. Mouse monoclonal antibody (mAb) clone 124 against Bcl-2, rabbit anti-mouse IgG-alkaline phosphatase conjugate, biotinylated rabbit anti-mouse antibody, avidin-biotin-peroxidase complex kit, and diaminobenzidine were purchased from DAKO (Copenhagen, Denmark). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

**FCM DNA analysis.**
The tissue sample was mechanically dissociated and the resulting cell suspension was processed for DNA analysis as described previously (23). Briefly fresh tumor tissue was...
mechanically dissociated in RPMI media and the resulting cell suspension was adjusted 1-2X10^6 nuclei/ml. Then it was stained with Coulter-DNA prep (100 μl of lyzing permeabilizing reagent (LPR) followed by 1ml staining solution (propidium iodide 50 u.g/ml + RNA Aase 4 KU/ml)). The solution was incubated in the dark for 60 minutes at room temperature. A Coulter profile II flow Cytometer was used to analyze 20,000 events after calibrating the diploid channel using propidium iodide stained tonsillar lymphocytes as the diploid control. DNA aneuploidy was defined as any population with a distinct peak corresponding to a DNA index (DI) > 1.05 or < 0.95 or the presence of tetraploid population greater than 15%. The cell cycle events were analyzed using micro-cycle soft developed by Peter S. Rabinovitch (Phoenix Flow systems, San Diego A).

**Preparation of cell lysates and measurements of Bcl-2 in cell lysates by EIA**

All steps of sample preparation were devised in our laboratory. All steps were carried out at 4 °C. Tissues were washed in ice-cold saline and homogenized and lysed on ice in an extraction buffer: 10 mmol/L (containing 10 mmol/L K_2 EDTA, 5 mmol/L mercaptoethanol, 0.39 mmol/L phenylmethylsulfonyl fluoride, and 5 mg/L aprotenin, pH 7.5) and processed as previously described (24). After centrifugation at 20000g for 20 minutes at 4°C, the supernatant (cell lysate) was frozen at -80 °C. We quantified the protein concentration in lysates by Bradford method (25), using bovine serum albumin as the calibrator. Bcl-2 antigen was measured in cell lysates with protein concentration adjusted to (1 g/L) according to the manufacturer's instructions. Briefly, the anti-Bcl-2 fluorescein isothiocyanate conjugate was applied to a microtiter plate coated with a mouse monoclonal antibody specific to human bcl-2 protein and then incubated with cell lysates, as well as with manufacturer-supplied kit calibrators (human Bcl-2 antigen at 0-400 kilounits/L). The resulting immune complexes were bound onto the plate, and any unbound reactants were removed by a washing step. Next, the wells with the bound immune complex were incubated with a linking solution (horse radish peroxidase labeled anti fluorescein isothiocyanate IgG) and then with a substrate-chromogen solution (3,3^5,5^ tetramethylbenzidine and hydrogen peroxide) to develop color. The color developing step was stopped by addition of 0.18 mol/L sulfuric acid. The intensity of color developed was read spectrophotometrically at 450 nm vs a substrate blank.

**Detection of Bcl-2 protein by western blotting technique**

Western blots were performed according to Sambrook et al. (26), and adapted by (15). Briefly, proteins from 60-μg cell lysates were separated by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The
gels were trans-blotted to NC filters in Tris- glycine buffer (25 mmol/L Tris, 192 mmol/L glycine, 20% methanol, pH 7.4) for 5 h at 60 V. The NC sheets were washed and the unoccupied binding sites were saturated with 5% bovine serum albumin in Tris-buffered saline (50 mmol/L Tris, pH 7.5, 150 mmol/L Nad) containing 2 mmol/L EDTA and 0.1% NP-40 for 1 h at room temperature and then were incubated with either anti-bcl-2 mAb or normal mouse IgG serum (negative control) overnight at 4°C. The membranes were washed with phosphate buffered saline containing 0.3% Tween. The antibodies bound to the NC membrane were visualized by incubation with rabbit anti-mouse IgG-alkaline phosphatase conjugate for 90 min at room temperature. Finally, the filters were incubated with alkaline phosphatase substrate solution 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium in 0.1 mol/L Tris buffer) at room temperature till the developed bands reached the desired intensity, and then the reaction was stopped by adding 200ul of 0.5 mol/L EDTA (pH 8.0) and 50 mL of phosphate-buffered saline. Comparison of the NC results with those in which normal mouse IgG serum was substituted for Bcl-2 mAb permitted identification of the Bcl-2 band.

Detection of Bcl-2 protein by Immunodot Blot

Immunodot blot was done according to Hawkes (27), and adapted by (15)as follows. NC sheet was wetted in PBS-tween 20 and loaded onto a bio-dot apparatus. Vacuum was applied for 5 min. to mark the wells onto the NC. Then, 20 ug lysates protein were added to each well and incubated in PBS-0.3% tween 20 containing 3% BSA for one hour at room temperature (RT). The NC was soaked in anti-Bcl-2 monoclonal antibody (MAOB), diluted 1:3000 in 1% BSA-PBS, for overnight at 4°C and then was washed 3 times in PBS-tween 20. NC was immersed in an alkaline phosphatase conjugate rabbit antimouse IgG at a dilution 1:5000 for 90 min. at RT, and then washed 3 times in PBS-tween 20. NC was immersed in the substrate solutions (1ml substrate buffer: 100 mM NaCl, 5 mM MgCl2, 100 mM Tris, pH 9.5; with 4 ul nitroblue tetrazolium and 4 ul 5-bromo-4-chlor-3-indolyl-phosphate) for 5-10 min. at RT.

Measurement of apoptosis (Cell Death) in cell lysates by EIA

The assay is a quantitative sandwich-enzyme-immunoassay-principle (Boehringer Mannheim, Germany) using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. The sample cell-lysate is placed into a streptavidin-coated MTP(microtiter plate) Subsequently, a mixture of anti-histone-biotin and anti-DNA-POD (peroxidase conjugated) are added and
incubated for 2 hours. During the incubation period, the anti-histone antibody binds to the histone-component of the nucleosomes and simultaneously fixes the immunocomplex to the streptavidin coated MTP via its biotinylation. Additionally, the anti-DNA-POD antibody reacts with the DNA-component of the nucleosomes. After removal of unbound antibodies by a washing step, the amount of nucleosomes is quantified by the POD retained in the immunocomplex. POD with ARTS (2,2'-Azino-di[3-ethylbenzthiazolin-sulfonat]) as substrate, and is determined photometrically at 405 nm, against substrate solution as a blank reference.

**Measurements in subcellular tissue fractions: ER, PR in the cytosol, p53 in the nucleus and HER-2/neu in the membrane fraction**

The frozen tissue chunk was homogenized on ice in 1:10 w/v ice cold homogenization buffer [10 mm/L Tris buffer, pH 7.5; containing 10 mmol/L K$_2$EDTA, 10mM/L glycerol, 5mmol/L benzamidine, 10mm/L Triton X-100, 10mm/L 2-mercaptopoethanol, 0.39 mm/L phenylmethylsulfonyl fluoride (PMSF), and 5 mg/L aprotonin] and recentrifuged at the same speed for additional 30 min. The protein concentration was determined by Bradford method (25), and HER-2/neu was measured in the membrane extract according to the Oncogene Science, manual and it was expressed as HNU/ug tissue protein where HNU is an arbitrary unit reflecting the number of molecules having binding sites for both monoclonal antibodies used in the assay (1 HNU=400ng).

In the cytosol (supernatant), the protein concentration was adjusted to 1-2 mg/ml and then ER and PR were measured according to Abbott enzyme immunoassay method (Chicago, USA). The crude nuclear pellet was dissolved in ice cold 10mmol/PBS(phosphate buffered saline; pH 7.2) by sonication for three bursts of 30 seconds each and centrifuged at 800 g for 15 minutes at 4°C. The washed nuclear pellet was re-suspended in 5 volumes ice cold swelling buffer(homogenization buffer containing 0.4 M KCL) on ice for 30 minutes with vortexing every 10 minutes. Thereafter, it was ultracentrifuged in Beckman LS
ultracentrifuge at 100,000 g for 30 minutes at 4°C. The nuclear extract (supernatant) was obtained, the total protein concentration was determined in the nuclear extract using Bradford method, and then, p53 was measured according to the Oncogene Science manual.

**Statistical analysis**
A multivariate analysis based on unconditional logistic regression (28) was made to investigate the prognostic role of different variables. Univariate analysis were performed using a Chi-square test of association or Fishers exact model to test the association of categorical variables, whereas t test was used for continuous variables. Kaplan Meier curves were created for those variables found to be significant in the multivariate analysis (29). Cut-off values were determined using receiver operating characteristic curves (30). All analyses were performed using statistical package for the social science (SPSS) on an IBM, PC Computer.

**Results**
The study included 94 breast cancer patients. All the patients in this study (94) had invasive duct carcinoma. During the follow-up period (up to 37 months), 34 patients (36.1 %) relapsed. Fifty cases (53.2%) were in stage I-n, whereas forty four cases (46.8%) were in , stage in-IV./ A total of 74 tumors (78.7%) had grade I-II, and 20 tumors (21.27%) had grade III. Fifty cases (53.2%) had no lymph node metastases, while 44 cases (46.8%) had lymph node metastases.

**Cutoff points for HER2/neu, Apoptosis, Bcl-2, p53, ER and PR:**
The best cutoff values were estimated to maximize the sum of sensitivity and specificity. They were (1.3 HNU/ug protein), (0.19U/ug protein), (15.1 U/mg protein) and (0.5ng/mg protein) for HER2/neu, apoptosis, Bcl-2 and p53 respectively, were calculated using ROC (Receiver Operating Characteristic) curve. While, cutoff points of 15 fmol/mg protein for ER, and PR were based on previous reports (31,32).

**The Univariate analysis of risk factors for relapse:**
The results of the univariate analysis are in Table I. All the clinicopathological features (the histological grade, tumor stage, and lymph nodes), and the genetic markers, HER2/neu, and mutant p53 were significantly associated with recurrent disease.

**The Multivariate analysis:**
The results of the Multivariate analysis depicted in Table II. Tumor stage, lymph nodes, and HER2/neu, were significant variables, predicting the recurrence of the tumor. While the tumor grade and p53 were nearly significant.

**Correlation between the molecular markers and the time of relapse in Grade subgroup:**
Bcl-2 was inversely correlated with the disease-free interval (DFI) in low grades(I,II tumors), while cell death was
correlated with DFI in high grade (III) as shown in Table III.

**Confirmation of Bcl-2 expression by WE and Immunoblot:**
Overexpression of Bcl-2 measured by EIA was confirmed by WB, and Immunodot blot in tissue samples(Figures 1,11).

**Kaplan Meier curve for HER2/neu:**
Is shown in (Figure III) as HER-2/neu > 1.31 cases had worse prognosis (DFS) than HER2/neu ^ 1.31.

**Discussion:**
A wide variety of morphology and molecular based prognostic factors and tumor markers have been studied due to their potentials, to predict disease outcome in breast cancer (33, 34, 35), but the results of breast cancer outcome studies were not uniform and there was inconsistent success at predicting disease prognosis.

To determine the significance of molecular-genetic prognostic factors, we performed a multivariate logistic regression analysis. Our results implied that HER-2/neu, tumor stage, and lymph nodes status were significant independent variables.

We did not find flow cytometry DNA pattern, a significant predictor for relapse. An adequate literature data base has established that operable breast cancers with a DNA index of 1.0 have a favorable prognosis but the DNA index fails to achieve independent prognostic significance using multivariate analysis because of correlations with more powerful prognostic factors (36).

An abundance of the literature is available describing the prognostic value of ER and PR (37,38). In this short-term study, ER and PR were not significant predictors for early relapse although they were the first known cell biologic prognostic factors predicting overall patient survival (39).

Apoptotic cell death in solid tumors is a subject of considerable interest in the last few years (40). The precise quantitation of apoptotic cells is necessary to determine the role of apoptosis in cancer growth, prognosis, and treatment. So, we assessed in this study, the extent of apoptosis which was determined using quantitative EIA technique. Our data showed that apoptosis was particularly prominent in high grade relapsed tumors where increased proliferative activity was evident. These results imply that proliferating tumors appear to have"high cell turnover state" in which there may be an increase chance of apoptosis amongst the proliferating cells. Dive and Wyllie (41), also suggested that, in high tumor grade, a high cell turnover with cell proliferation exceeding the rate of cell death was found, and may also overcome the protective effect of cell survival factors such as the anti-apoptotic proteins as Bcl-2.

In our series, Bcl-2 protein was measured in a tissue sample by quantitative EIA method and the results
were confirmed by WB and immunodot blot. Fifty-five percent of breast carcinoma were elevated above the determined cutoff value (15.1 U/mg protein), confirming the previous reports detected by immunohistochemical method (42, 43, 44, 45). Some studies demonstrated significant correlations between Bcl-2 gene expression and recurrence (44, 46, 47, 48), but in our study, bcl-2 was inversely related to the disease-free survival only in low grade tumors where decreased proliferative activity was evident, indicating that it could be an early event of tumorigenesis, and strongly correlated with a bad prognosis.

A number of immunohistochemical studies have found that alterations in p53 protein are associated with a worse prognosis (49). In the present study, p53 measured by EIA method was an independent predictor for early recurrence, but less significant than HER-2/neu. (16, 50).

Many literatures are available, describing the association of HER-2/neu with increased risk of relapse and death (20, 51, 52, 53, 54). To the best of our knowledge this study is among the first to investigate this panel of molecular prognostic markers in breast cancer in a multivariate analysis. HER-2/neu was the strongest independent parameter for early relapse due to increased metastatic potential which is a consequence of the motility enhancing activity of the HER-2/neu protein, which is exclusively expressed on pseudopodia, and to a lesser extent of its growth stimulating effect (55).

From clinical point of view, the assessment of HER-2/neu over-expression in breast cancer might become a useful tool in the future in the treatment of patients not only with chemotherapy, but also with MAB HER-2 (Herciptin) directed towards Her-2/neu protein.

**Acknowledgment:** The authors thank the Surgical and Pathology Departments, Ain Shams Faculty of Medicine for providing surgical specimens and information regarding surgical-pathological features of the tumors.
**Table I: Univariate analysis of risk factors for early relapse in breast cancer:**

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**Figure Legend:**

**Figure I:** Western blotting analysis of Bcl-2 in neoplastic breast tissues. Expression of Bcl-2 in breast cancer (lanes 1-5). Lane 5, molecular weight standards. Note that in all malignant breast samples, a single band of approximately 26 kD, which corresponds to the Mwt of Bcl-2 protein was recognized, although the intensities of the band varied according to Bcl-2 concentration.

**Figure n:** Immunodot blot of malignant breast tissues. Lanes 2-3 show dense color intensities according to Bcl-2 concentrations. Lane 1 negative control (Bcl-2 MOAB is substituted by normal mouse serum).

**Figure m:** Kaplan-Meier survival curves according to HER-2/neu expression in neoplastic breast tissues. Patients with HER-2/neu>1.31 HNU/ug protein had significantly lower rate of disease free survival than did those with HER-2/neu<1.31 HNU/ug protein.
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استخدام التحليل الاحصائي التعددي لمعرفة دور الدلائل المختلفة (bcl2 – Apoptosis, P53, Her-2neu) سرطان الثدي مع فترة قصيرة للمتابعة

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قسم الكيمياء الحيوية بكلية طب عين شمس
قسم الكيمياء الحيوية بمعهد البحوث
قسم علاج الأورام بالاشعة والطب النووي بطب عين شمس

الملخص العربي

يوجد الكثير من التغييرات الجينية في الخلايا في حالات سرطان الثدي تشتمل التغيرات التكوينية في الخلايا النموية وتعديل في وظائف الخلايا المبرمج في وظيفة P53 وكذا، وهي جميعا تكون مصحوبة للحالات ذات الوضع السئ لحالات مرضي سرطان الثدي.

ولقد استخدم التحليل الاحصائي التعددي لمقارنة تلك الدلائل مع العوامل السابق معرفتها مثل مرحلة تواجد المرض والتحليل البيولوجي وغيرها.

تحتوي هذه الدراسة علي اربع وتسعة عينة طارئة من النسيج المصيب من الورم السرطاني بالثدي حيث تم دراسة الدلائل السابق ذكرها بكل من جهاز دراسة تدفق الحمض النووي والهرمونات كذلك والألبروك وغيرها من الطرق البيولوجية الحديثة المختلفة.

كما تم دراسة التحليل البيولوجي وطرق العلاج المختلفة للحالات المشتركة في البحث.

ولقد وجد أن درجة نشاط الورم، مدى انتشار الورم، وتاليه العدد الليمفاوية مع تواجد خلل في الدلائل B53، Her 2 neu تكون بإستجابة سلبية للعلاج وكل منها دلالة مندزة مستقبلية بالتحليل الاحصائي.

وتكون درجة نشاط الورم مع سرعة ارتفاع الورم تنتج عنه إصوص Bcl2 يعتبر دلالة هامة لمرضي سرطان الثدي ذو الدرجة القليلة لنشاط الورم بينما يطرح موت الخلايا يعتبر داللة هامة في الورم عالية النشاط.

ولذا، فقد تمت من التحليل البيولوجي الخلايا الجينية 1~2 ب يعتبر من أقوي Her-2 neu +ve، والدلائل المذكورة بسواء الاستجابة القصيرة المدى للعلاج لمرضي سرطان الثدي.

وإن مرضي سرطان الثدي مع (Her-2 new +ve) قد تتحسن الاستجابة للعلاج مع استخدام عقار (هيرستين).