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## **QUANTITATIVE ANALYSIS OF C-ERBB2 AND P53 PROTEIN LEVELS IN BREAST TUMOR TISSUES AND STUDY OF THEIR ASSOCIATIONS WITH TUMOR BIOLOGICAL CHARACTERISTICS AND PROGNOSIS**

(With 10 Tables and 2 Figures)

By

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**التحليل الكمي لمستويات بروتين سرب ب ٢ وب ٥٣ في أنسجة أورام الثدي  
ودراسة العلاقة بينهم وبين الخصائص الحيوية والتطورات المصاحبة للورم**

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يعتبر سرطان الثدي من الأمراض متعددة الطبائع وأن سلوكها غير متوقع. وحديثاً قد توجهت الاهتمامات ناحية بعض الدلالات الحيوية لسرطان الثدي التي تعطي معلومات قيمة عن تطورات هذا المرض. من بين هذه الدلالات بروتين سرب ب ٢ وب ٥٣. والغرض من هذه الدراسة هو قياس مستويات بروتين سرب ب ٢ وب ٥٣ في أنسجة أورام الثدي وكذلك دراسة العلاقة بين مستويات هذه الدلالات والسلوك الأكلينيكي والمرضي للورم. وقد تم قياس مستويات بروتين سرب ب ٢ وب ٥٣ في أنسجة ورم ثدي سرطاني لعدد ٤٠ مريضة وأنسجة ورم ثدي حميد لعدد ٢٠ مريضة وأنسجة ثدي غير مصابة لعدد ١٥ مريضة وهذه الأنسجة قد أخذت أثناء عمليات استئصال الثدي أو استئصال ورم من الثدي. وقد أظهرت نتائج هذه الدراسة وجود زيادة ذات قيمة في مستويات بروتين سرب ب ٢ وب ٥٣ في أنسجة الورم السرطاني مقارنة بأنسجة الورم الحميد أو أنسجة الثدي الغير مصابة. وهذه الزيادة لها علاقة مع المرحلة المتقدمة وعدد الغدد الليمفاوية وحمولة الورم وسرطان الأوعية اللمنية وكذلك التطورات السيئة للمرض. وكما وجدت علاقة طردية بين مستويات سرب ب ٢ وب ٥٣. والخلاصة اعتماداً على هذه النتائج يتضح أن سرب ب ٢ وب ٥٣ يعتبروا من الدلالات الهامة التي تساعد على توقع السلوك الحيوي لسرطان الثدي. لذلك توجيه الوسائل العلاجية ناحية الجينات النشطة الخاصة بهذه الدلالات في هذه النوعية من المرضى من الممكن أن تؤدي إلى نتائج علاجية لهذا المرض.

### **SUMMARY**

Breast cancer is a disease with heterogenous nature. Its behavior is unpredictable. There has recently been much interest in the biological

parameters for breast cancer as they may provide useful information on the clinical outcome. Among these parameters c-erbB2 and p53 are of special importance. The present study was designed to determine the protein levels of c-erbB2 and p53 in breast tumor tissues by ELISA technique correlating the levels with clinicopathological behavior of tumors. C-erbB2 and p53 protein levels were determined by ELISA technique in 40 malignant breast tissues, 20 benign breast disease tissues and 15 healthy breast tissues. These tissues were obtained during operations of mastectomy and lumpectomy. C-erbB2 and p53 protein levels were significantly increased in malignant breast tissues compared to either benign or healthy tissues. The levels were correlated with advanced stage, number of lymph nodes involved, tumor burden, ductal carcinoma and bad prognosis. Significant positive correlation ( $r=0.72$  &  $P < 0.001$ ) existed between p53 and c-erbB2 protein levels. Based on these finding c-erbB2 and p53 are important predictors of breast cancer biological behavior. Therapeutic modalities directed against these genes in patients over-expressing them could be of great therapeutic results.

**Key words:** *Breast cancer, ELISA, tissue protein levels*

## INTRODUCTION

Breast carcinoma is the most common cause of cancer among women worldwide. Incidence rate are high in more developed countries whereas in less developed countries are low but increasing. In Egypt, breast cancer is the most common cancer among women, representing 18.9% of total cancer cases among the Egypt National Cancer Institute (NCI) series of 10556 patients during the year 2001 (El-atar, 2001). It is a pathologically and clinically heterogenous disease with variable prognosis. Breast carcinoma is potentially highly malignant tumors due to their ability to invade locally and metastasize. In spite of this tendency, still more women are living with breast cancer due to better mamogoraphy and the development of adjuvant therapy (Wurtz *et al.*, 2005).

In order to understand the biological behavior of breast cancer, its genetic bases should be studied. A number of genes whose abnormalities contribute to tumorigenesis have been identified. They can be divided into three groups, namely, oncogene, tumor suppressor genes and DNA repair genes.

The HER-2/<sup>neu</sup> (c-erbB2) gene is localized to chromosome 17 g and encodes a transmembrane tyrosine kinase receptor protein, that is a

member of epidermal growth factor receptor (EGFR) (Navolanic *et al.*, 2003 and Ross *et al.*, 2004). This family of receptor is involved in cell-cell and cell-stromal communication primarily through a process known as signal transduction, in which external growth factors, or ligands, affect the transcription of various genes by phosphorylating or dephosphorylating a series of transmembrane proteins and intracellular signaling, many of which possess enzymatic activity. Signal propagation occurs as the enzymatic activity of the next protein in the pathway (Karungaran *et al.*, 1996), ultimately affecting cell proliferation, survival, motility and adhesion (Ross *et al.*, 2004). Receptor activation requires three variables, a ligand, a receptor dimerization partner. After a ligand binds to a receptor, that receptor must interact with another receptor of identical or related structure in a process known as dimerization, in order to trigger phosphorylation and activation signaling cascades. Therefore, after ligand binding to an EGFR family member, the receptor can dimerize with various members of the family. It may dimerize with a like member of her family (homodimerization), or it may dimerize with a different member of the family (heterodimerization). The specific tyrosine residues on the intracellular portion of HER-2/<sup>neu</sup> receptor that are phosphorylated, and hence the signaling pathways that are activated depends on the ligand and dimerization partner. The wide variety of ligands and intracellular cross talk with other pathways allows for significant diversity in signaling (Yarden and Sliwkowski, 2001 and Ross *et al.*, 2004). While no known ligand for the HER-2/<sup>neu</sup> receptor has been identified, it is the preferred dimerization partner of the other family members. HER-2/<sup>neu</sup> heterodimers are more stable (Tzahar *et al.*, 1996 and Ross *et al.*, 2004) and their signaling is more potent (Carven *et al.*, 2003) than receptor combination without HER-2/<sup>neu</sup>. The HER-2 alteration, which is gene amplification and resulting in over expression, leading to increased circulating HER-2/ neu receptor protein levels serves as a tumor marker. These levels have successfully predicted the presence and progression of HER-2/<sup>neu</sup> positive breast cancer. Thus, according to Ross *et al.* (2004), in his review, in 20 published studies, some studies reported a significant correlation of serum HER-2/<sup>neu</sup> protein levels with disease recurrence, metastasis or shortened disease-free and overall survival (Revillon *et al.*, 1996) in women with either lymph node- negative (Press *et al.*, 1997) or lymph node- positive breast cancer (Gusterson *et al.*, 1992). Other studies reported no significant association of serum levels with prognosis (Volas *et al.*, 1996).

HER-2/<sup>neu</sup> gene amplification and protein over expression have been consistently correlated with high tumor grade, high cell proliferation rate negative assay for nuclear protein receptors for estrogen and progesterone, p53 mutations, topoisomerase IIa amplification, and alteration in a variety of biomarkers of breast cancer invasiveness and metastasis (Navolanic *et al.*, 2003 and Zemzoum *et al.*, 2003). Moreover, HER-2/<sup>neu</sup> gene amplifications appears to be strongly correlated with tumor grade and ductal versus lobular status (Mack *et al.*, 1997 and Rosenthal *et al.*, 2002). HER-2/<sup>neu</sup> over expression has been a consistent feature of both mammary and extramammary Paget's disease (Hanna *et al.*, 2003). HER-2/ neu status had been found to be consistent primary tumors and their corresponding metastasis (Symmans *et al.*, 1995). According to Ross *et al.*, (2004) in his review article, low level HER-2/<sup>neu</sup> over expression has been identified in benign biopsies and associated with an increased risk of subsequent invasive breast cancer (Stark *et al.*, 2000). Serum HER-2/<sup>neu</sup> protein levels were tested for their ability to predict response to therapy, according to Harris *et al.* (2001) and Lipton *et al.* (2002), elevated serum HER-2/<sup>neu</sup> protein level predicted therapy resistance. However, Revillion *et al.* (1996) and Volas *et al.* (1996) had not found this association. Moreover, serum HER-2/<sup>neu</sup> levels were found to be correlated with absence of clinical response to hormonal therapy in estrogen receptor (ER)-positive tumors (Lipton *et al.*, 2002), but not in others (Volas *et al.*, 1996). Serum HER-2/<sup>neu</sup> have successfully predicted resistance to high-dose chemotherapy (Harris *et al.*, 2001) and response to trastuzumab (Herceptin), a recombinant humanized monoclonal anti-HER-2 antibody single agent and combination treatment for metastatic HER-2/<sup>neu</sup> positive disease (Ali *et al.*, 2002).

Among the molecular genetic alterations that had been identified in human breast cancer is alteration in p53 function. P53 is encoded by the Tp53 gene. located at 17p13, this contains 11 exons spanning 20 kb. It belong to a family of highly conserved genes that also includes TP63 and TP73, encoding p63 and p73 respectively. P53 protein is a nuclear phosphoprotein, consisting of 393 amino acids with a half-life of about 25 minutes (Levine *et al.*, 1992). While p53 seems to be dispensable for normal development (Donehower *et al.*, 1992), it plays an important role in regulating cell fate in response to various stresses, either genotoxic (DNA alterations induced by irradiation, UV, carcinogens and cytotoxic drugs) or non genotoxic (hypoxia, nucleotide depletion, oncogene activation, microtubule disruption, loss of normal cell contacts). The

protein may be viewed as a node for the stress signals, which are then transduced, mainly through the ability of p53 to act as a transcription factor. P53 exerts its anti-proliferative action by inducing reversible or irreversible (senescence) cell cycle arrest, or apoptosis. It may also enhance DNA repair and inhibit angiogenesis (Lacroix *et al.*, 2006). Many types of stresses may be encountered during tumor development. The p53 function is often altered in cancer. It has been suggested that p53 could have evolved in higher organisms specifically to prevent tumor development (Vousen and Lu, 2002). IT is believed that this specific action is exerted mainly through the triggering of apoptosis (Yu and Zhang, 2005). Indeed loss of p53 activity disrupts apoptosis and accelerates the appearance of tumors in transgenic mice (Attardi and Jacks, 1999).

The qualitative and quantitative activity of p53 depends on its integrity (mutation status), its amount, and its specific posttranslational modifications induced by the activation of the different stress-induced signaling pathways. This leads to variable patterns of association between p53 and a number of other co-regulatory proteins, of which some may be tissue-or cell type specific. Thus, p53 activity is controlled in a very complex manner, including several auto-regulatory loops, through the intervention of dozens of modular proteins (the p53 interactome) P53 is subject to tight regulations at multiple levels. In cancer cells, its functions can be compromised by various mechanisms: mutations of Tp53, alteration of p53 regulators, alteration of p53 target genes (intractome components).

In humans, inheritance of Tp53 mutant allele results in a rare familial autosomal disorder, the Li-Fraumeni syndrome. However, most p53 mutations, observed in breast cancer are of somatic origin. Between 20 and 35% of breast tumors have been shown to express a mutant p53 (Vousden and Lu 2002). The majority of p53 mutations appear to be localized in the DNA-binding domain, in the central part of p53. Notably, this domain is the binding site for important cofactors in the transactional activity of p53 in relation to apoptotic genes (Lacroix *et al.*, 2006). Since there is no evidence that Tp53 lies in a hyper-mutable region of the genome, cells that have lost p53 function are likely to be selected during cancer development. In cells expressing a mutant p53, this protein is generally no longer able to control cell proliferation, which results in inefficient DNA repair and genetic instability. The great majority of mutant p53 are defective in transactivation and may exert a dominant negative effect by preventing wild-type p53 from binding to

the promoter of its target genes (Willis *et al.*, 2004). Contrasting with other tumor-suppressor genes, p53 mutants are most frequently (~90%) missense (point missense mutations). Mutant p53 proteins generally have an increased stability and accumulate in the nucleus of neoplastic cells. It is believed that this is a consequence of the p53 mutant protein no longer deriving the expression of MDM2 protein required to target its own degradation (Lacroix *et al.*, 2006). Immunohistochemical detection of the amount of nuclear p53 has long been used as an indicator of p53 alteration, but this parameter appears highly dependent on type of mutation. The potential relationship between p53 alteration and the expression of other tumor markers or pathological characteristics (grade) have been widely investigated. Breast tumors expressing a high amount of p53 (as measured by immunohistochemistry) are more frequently ER-negative and progesterone receptor (PgR)-negative. They are also associated with a high proliferation rate, high histological and nuclear grades, aneuploidy and poorer survival. A high p53 level is frequently observed in tumors over-expressing c-erb B-2 (Feki and Irminger-Finger, 2004 and Lacroix *et al.*, 2006). The same relationships have been found considering p53 mutations rather than accumulation. Thus, according to Bull *et al.* (2004) patient with node-negative breast cancer, p53 mutations were more frequent in breast carcinomas with amplification of c-erb B2 gene thus, leading to accumulation of c-erbB2 over-expression. Patients with both p53 mutation and c-erbB2 amplification were associated with poor survival. According to Tsutsui *et al.* (2003), breast cancer with a coexpression of p53 protein and c-erbB2 have a more significant prognostic value than those with a positive expression of either of these two biological parameters, which themselves remained independent of each other. Moreover, the particularly bad prognosis associated with coexistence of p53 alterations and high c-erbB2 was also reported by Rahko *et al.* (2003) and Yamashita *et al.* (2004).

Since p53 mutations and c-erbB2 amplification are the most common abnormalities in the breast cancer and due to the controversial reports regarding their role as diagnostic and prognostic value, the present study was performed to analyze the protein levels of p53 and c-erbB2 by ELISA in breast cancer cytosols. The levels of these important biological parameters would be correlated with tumor characteristics and prognosis.

## **MATERIALS and METHODS**

### **Patients:**

The present study included 40 patients with malignant breast lesions and 20 patients with benign breast lesions. These patients were admitted to surgery department, south valley cancer institute hospital. All patients were subjected to thorough clinical assessment in the form of personal (including family history of similar conditions), menstrual, gynecological and obstetric history taking as well as presenting symptoms. Each patient was examined both generally and locally for the mass of the breast in the form of its size, location, consistency, bilaterality, skin and nipple changes. Radiological examinations in the form of chest x-ray, mamography, sonography and CT or MRI when required was performed. Routine laboratory investigations in the form of complete blood picture, liver and kidney functions as well as random blood sugar was also done for each participant.

### **Methods:**

During operation, tissue samples, free fat, necrotic material and nascent blood (250 -750 mg) were obtained. The tissues were pulverized while frozen in a chilled mortar and homogenized for 2 minutes in TED buffer, (Tris 10 mM, EDTA disodium salt 1.5 mM and Dithiothreitol 1 mM, pH 7.4 at 4°C) in melting ice using a teflon homogenizer. These homogenates were centrifuged for 10 minutes at 10,000Xg at 4°C. The supernatants were recentrifuged at 3000 Xg for another 15 minutes at 4°C. Finally, the resultant supernatants were stored at -70°C in 200 ml aliquots till assay.

Tissue levels of c-erbB2 were determined by ELISA, using the human neu oncoprotein ELISA kit supplied by Oncogene Science Inc., OIA-O4, Uniondale, New-York. For tissue homogenates 100 µl of tissue homogenate was extracted with 20 µl of antigen extraction agent supplied with the kit. The tubes were centrifuged at 1500 Xg for 10 minutes. The supernatants were diluted 50 times with sample dilution buffer supplied in the kit. Then, 100 µl of diluted sample were used for the determination of Her-2/neu as exactly described in the booklef of the kit.

Tissue levels of p53 were also determined by ELISA technique, using kits suppllied by Diaclone Research, France, Cat. No. 043. A monoclonal antibody specific for p53 has been coated onto the wells of the microtiter strips provided. During the first incubation, the p53 antigen is added to the wells. After washing, a biotinylated monoclonal antibody specific for p53 is incubated. Then, the enzyme (streptavidin-

peroxidase) is added. After incubation and washing to remove all unbounded enzymes, a substrate solution, which acts on the bound enzyme is added to induce a colored reaction product. The intensity of this colored product is directly proportional to the concentration of p53 present in the samples.

Protein concentration in the tissue homogenates was determined by the method of Lowry *et al.* (1951) as modified by Miller (1959) which combines the Biuret and Folin Ciocalteu reactions (Henry *et al.*, 1974). For comparative analysis 15 healthy tissues were obtained during cosmetic breast operation.

## RESULTS

The clinical criteria of patients are shown in Table (1). The levels of c-erbB2 and P53 in control tissues, benign breast diseases and malignant breast tissues are shown in Table (2). The levels of these two proteins were significantly higher in tissues of patients with benign breast diseases in compared to control tissues. Moreover, tissues of patients with malignant breast diseases showed significantly higher levels of both c-erbB2 and P53 compared to either tissues of patients benign breast diseases ( $p < 0.001$ ) or healthy tissues ( $p < 0.001$ ). The difference between the three groups is farther clarified from the scatterogram showing individual levels (Fig. 1 and 2).

Table (3) and (4) exhibits the relationship between menopausal status and cerb B-2 as well as P53 in both benign and malignant breast tissues. While no significant effect of menopausal status could be observed on the levels of these two proteins in benign breast diseases tissues, the levels were higher in postmenopausal breast cancer patients tissues, but the difference was significant in case of c-erbB2 ( $p < 0.05$ ). Table (5) shows the effect of stage of disease on indices studied. Patients with stage III & IV showed significantly higher levels of both c-erbB2 and P53 compared to stage I & II ( $p < 0.001$  for each).

Patients with more than two lymph nodes involvement showed significantly higher levels of c-erbB2 and P53 compared to patients with one or two lymph nodes involved ( $p < 0.001$  for each) (Table 6). As evident in Table (7), patient with high tumor burden also exhibited significantly higher levels of these indices. Also, Patients with infiltrating ductal carcinoma showed significantly higher levels of c-erbB2 and P53 compared to patients with other pathological types ( $p < 0.001$  for each) (Table 8). Meanwhile, patients with good prognosis as determined by four years disease free survival showed significantly higher levels of c-erbB2 ( $p < 0.01$ ) and P53 ( $p < 0.001$ ) (Table 9).



Correlation between the two indices in benign breast tissues is shown in Table.(10). A significant positive correlation was observed ( $r = 0.182$  &  $p < 0.001$ ). Meanwhile the correlation between c-erbB2 and P53 protein levels in malignant breast tissues is clarified in Table (10). A significant positive correlation was observed between the two indices ( $r = 0.72$  &  $p < 0.001$ ).

**Table 1:** clinical criteria of patients of benign as well as malignant breast lesions.

	Benign breast lesions (n=20)	Malignant breast lesions (n=40)
Age	Range 17 – 65years Means±S.E 34.2±	Range 26 - 70 years Means±S.E 45.62±1.9
Pathology:	Fibrocystic disease:8 Fibroadenoma: 5 Duct ectasia: 3 Chronic abscess: 2 Lactating adenoma: 2	Infiltrating duct carcinoma:29 Ductal carcinoma insitu:4 Fibroadenocarcinoma:3 Lobular carcinoma:2 Lobular carcinoma insitu:2
Staging:	—	Stage I&II: 23 Stage III&IV: 17
Lymph node status:	—	N0:6 N1:15 N2:10 N3:9
Menopausal status:	Premenopausal 17 Postmenopausal 3	Premenopausal 24 Postmenopausal 16
Size of the mass:	<25 cm 16 >25 cm 4	<25 cm 14 >25 cm 26

**Table 2:** C-erbB2 and P53 levels in patients' tissues with benign breast lesions, malignant breast lesions compared to controls' tissues.

Group		C-erbB2 (HNU/μg)	P53 (U/mg)
Control (n=15)	Mean ± SE	0.45±0.12	0.85± 0.18
	Median	0.37	0.72
	Range	0 – 1.35	0 – 2.35
Benign breast diseases (n=20)	Mean ± SE	1.75±0.18	2.32±0.23
	Median	1.12	2.11
	Range	0.38-5.61 P<0.001	0.0 – 6.11 P<0.001
Malignant breast diseases (n=40)	Mean ± SE	4.76±0.64	10.46±1.41
	Median	4.32	7.51
	Range	0.8 – 12.43 P<0.001 P*<0.001	2.39 – 30.95 P<0.001 P*<0.001

N.B: statistical analysis was performed by Mann-Whitney test

Versus control tissues

\*Versus benign breast disease tissues

**Table 3:** C-erbB2 and P53 levels in tissues of premenopausal and postmenopausal patients with benign breast lesions.

Group		C-erbB2 (HNU/ $\mu$ g)	P53 (U/mg)
Premenopausal patients with benign breast disease (n=17)	Mean $\pm$ SE	1.73 $\pm$ 0.18	2.32 $\pm$ 0.24
	Median	1.12	2.1
	Range	0.38 - 5.61	0 - 6.11
Postmenopausal patients with malignant breast disease (n=3)	Mean $\pm$ SE	1.42 $\pm$ 0.41	1.79 $\pm$ 0.63
	Median	1.11	1.69
	Range	0.53 - 3.58	0 - 4.56
		N.S.	N.S.

**Table 4:** C-erbB2 and P53 levels in tissues of patients with breast cancer as a function of menopausal status.

Group		C-erbB2 (HNU/ $\mu$ g)	P53 (U/mg)
Premenopausal breast cancer tissues (n=24)	Mean $\pm$ SE	3.60 $\pm$ 0.71	9.19 $\pm$ 1.78
	Median	2.36	5.96
	Range	0.71 - 10.54	2.39 - 30.95
Postmenopausal breast cancer tissues (n=16)	Mean $\pm$ SE	6.19 $\pm$ 1.11	12.07 $\pm$ 2.49
	Median	6.61	8.34
	Range	1.74 - 12.43	3.82 - 30.22
		P<0.05	N.S.

**Table 5:** Tissular C-erbB2 and P53 in breast cancer patients according to stage of disease.

Group		C-erbB2 (HNU/ $\mu$ g)	P53 (U/mg)
Stage I&II (n=23 )	Mean $\pm$ SE	2.79 $\pm$ 0.56	4.21 $\pm$ 1.05
	Median	2.18	5.55
	Range	0.8 - 7.13	2.39 - 14.97
Stage III&IV (n= 17)	Mean $\pm$ SE	6.82 $\pm$ 0.94	14.20 $\pm$ 2.45
	Median	7.42	12.91
	Range	0.93 - 12.43	3.82 - 30.92
		P<0.001	P<0.001

**Table 6:** Tissular levels of C-erbB2 and P53 in breast cancer patients as classified according to number of lymph nodes involved.

Group		C-erbB2 (HNU/ $\mu$ g)	P53 (U/mg)
1&2 lymph nodes (n=21 )	Mean $\pm$ SE	3.12 $\pm$ 0.67	5.16 $\pm$ 1.21
	Median	2.27	5.92
	Range	0.8 - 10.54	3.11 - 16.26
3&4 lymph nodes (n=19 )	Mean $\pm$ SE	6.99 $\pm$ 1.04	16.35 $\pm$ 3.11
	Median	7.24	13.12
	Range	1.7 - 12.43	4.1 - 30.15
		P<0.001	P<0.001

**Table 7:** Tissular levels of C-erbB2 and P53 protein levels as a function of tumor burden.

Group		C-erbB2 (HNU/ $\mu$ g)	P53 (U/mg)
Low tumor burden (n=18)	Mean $\pm$ SE	3.53 $\pm$ 0.72	7.42 $\pm$ 1.11
	Median	3.12	8.10
	Range	0.9 – 8.53	3.0 – 15.12
High tumor burden (n=22)	Mean $\pm$ SE	7.42 $\pm$ 1.11	17.11 $\pm$ 2.91
	Median	8.1	16.8
	Range	1.0 – 12.35	4.1 – 30.21
		P<0.001	P<0.001

**Table 8:** Tissular levels of C-erbB2 and P53 protein levels as classified according to pathology.

Group		C-erbB2 (HNU/ $\mu$ g)	P53 (U/mg)
Infiltrating ductal carcinoma (n = 29)	Mean $\pm$ SE	8.11 $\pm$ 1.21	18.32 $\pm$ 3.15
	Median	7.9	18.21
	Range	2.1 - 12.51	4.15 - 30.1
Other pathological types (n = 11)	Mean $\pm$ SE	3.91 $\pm$ 0.91	7.44 $\pm$ 2.17
	Median	3.75	7.12
	Range	1.1 - 7.21	2.2 - 15.83
		P<0.001	P<0.001

**Table 9:** Tissular levels of C-erbB2 and P53 protein levels classified according to prognosis.

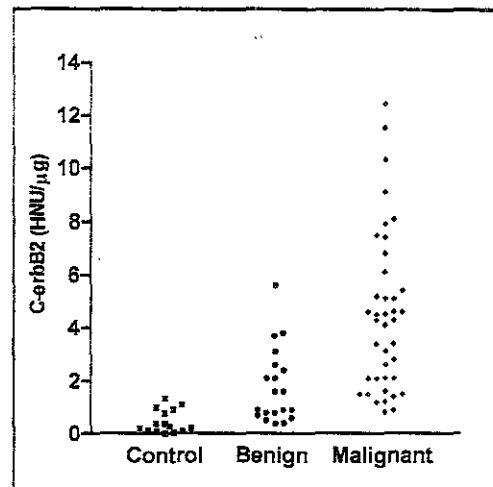
Group		Good prognosis (n=15)	Bad prognosis (n=25)
C-erbB2 (HNU/Mg)	Mean $\pm$ SE	4.13 $\pm$ 0.32	8.31 $\pm$ 1.5
	Median	3.9	8.2
	Range	0.8 – 8.21	1.3 – 11.7
P53 (U/mg)	Mean $\pm$ SE	6.35 $\pm$ 1.91	17.1 $\pm$ 2.21
	Median	7.0	16.9
	Range	3.5 – 21.6	5.1 – 29.6
			P<0.001

**Table 10:** Correlation between C-erbB2 and P53 in benign and malignant breast tissues.

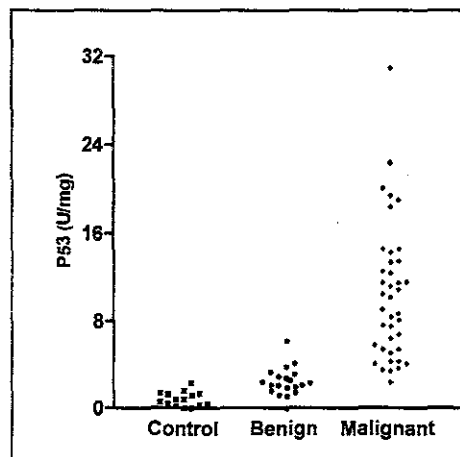
In benign breast tissues		In malignant breast tissues	
Variable	C-erbB2 (HNU/Mg)	Variable	C-erbB2 (HNU/Mg)
P53 (U/mg)	(0.182)	P53 (U/mg)	(0.72)
	<0.001		<0.001

Values between brackets are correlation coefficients (r)

Values without brackets are levels of significance (P)



**Fig. 1:** Individual values of c-erbB2 in control, benign and malignant groups.



**Fig. 2:** Individual values of P53 in control, benign and malignant groups

## DISCUSSION

Over expression of c-erbB2 receptor tyrosine kinase in breast cancers is associated with most aggressive tumors. It is able to confer many of the characteristics of a cancerous cell, including uncontrolled proliferation, resistance to apoptosis and increased motility. c-erbB2

over expression is specific to tumor cell (Badache and Goncalves, 2006). More than 15 years ago, seminal studies were reported that abnormal expression of c-erbB2 receptor tyrosine kinase in breast tumor was linked to poor prognosis. Due to controversial reports attributed to different techniques, the present work was performed using enzyme-linked immunosorbent assay (ELISA) technique which is performed on tumor cytosols made from fresh tissue samples that avoids the potential antigen damage associated with fixation, embedding and uncontrolled storage. ELISA-based measurements of HER-2/<sup>neu</sup> protein in tumor cytosols correlated closely with both HER-2/<sup>neu</sup> gene amplification results detected by FISH and protein expression results measured by immunohistochemical techniques (Muller *et al.*, 2003).

In the present study, we observed that patients with benign breast diseases had significantly higher levels of c-erbB2 protein levels in their cytosols compared to control tissues. The levels in malignant breast tissues were significantly higher than both benign breast disease or control tissues (Table 2 and Fig. 1). The present findings are in agreement with previous results. Thus, Kalogeraki *et al.* (1996), reported significantly higher c-erbB2 protein levels in patients with breast cancer compared with benign breast diseases.

According to Brower *et al.* (1995), levels of this oncoprotein could be useful in defining malignant and benign lesions. Thus the levels in the benign breast were < 10 HNU/ $\mu$ g, a finding also observed in the present study as evident in Fig. (1). Also Pleizzola *et al.* (1999), reported significantly higher levels of c-erbB2 in breast cancer compared to healthy and benign tissues.

Several studies have studied the levels of c-erbB2 protein in benign breast diseases as a risk factor for progression to breast cancer, but with controversial results.

Rohan *et al.* (1998), reported that c-erbB2 protein over expression appears to be not associated with an increased risk of progression to breast cancer. Millikan *et al.* (1995), studied cerb B-2 protein levels in patients with benign breast diseases that may be at increased risk for invasive breast cancer (lesion that exhibit atypical epithelial proliferation or fibroadenoma). They observed that low levels were exhibited in 8.3% of cases.

Stark *et al.* (2000) reported that patients with benign breast diseases who did not develop breast cancer during the follow up period, 4.5% of tissues demonstrated low levels of c-erbB2 amplification whereas 9.5% of benign breast disease exhibited amplification during follow up period.

C-erbB2 amplification in benign breast biopsies was associated with an increased risk of breast cancer.

This association approached statistical significance. For women having both c-erbB2 amplification and a proliferative histopathologic diagnosis (either typical or atypical).

Stark *et al.* (2000) reported that the risk was more than 7 fold, in patients with over-expression of the c-erbB2 in their prior benign breast biopsies. The highest value in benign breast diseases could be used as the threshold to distinguish between positive and negative breast cancer samples. In the study of Millikan *et al.* (1995), benign breast biopsies that exhibit atypical epithelial proliferation or fibroadenoma who are at increased risk for invasive breast cancer, do not show c-erbB2 amplification, but 8% showed low level immunoreactivity to c-erbB2 protein product.

Wells *et al.* (1995) in their study on patients with benign breast diseases associated with apocrine adenosis, reported c-erbB2 protein positivity in 57.1% in apocrine cells within sclerosing adenosis. Thus it seems likely that the expression of abnormal oncogene products and increased proliferation in some of these apocrine lesions questions the supposed degenerative nature of the atypia seen in such cases and suggests that there may be an association between these lesion and large cell ductal carcinoma in situ and hence invasive carcinoma (Wells *et al.* 1995).

Discordant reports to the present results and to previous investigators were reported by Lizard *et al.* (1995) and Zhang *et al.* (1997). Thus, Lizard *et al.* (1995), analyzed c-erbB2 gene amplification in a group of benign breast diseases including fibroadenomas, benign phyllode tumors and fibrocystic diseases. Non of these cases showed c-erbB2 gene amplification. These authors reported that the molecular alterations, which are more frequently involved in malignant breast carcinoma do not occur in benign breast diseases. According to Zhang *et al.* (1997) all benign breast diseases were negative for c-erbB2 overexpression. As also observed in the present study, Rohan *et al.* (1998), reported that protein levels of c-erbB2 did not differ among strata defined by menopausal status (Table 3).

The significantly increased levels of c-erbB2 in breast cancer compared to either benign breast cancer or controls were also reported by Regidor *et al.* (1995) and Plezzola *et al.* (1999), using ELISA technique. Using the highest level of c-erbB2 in benign breast diseases tissues as a cut off value, (5.6 HUN/ $\mu$ g), 11/40 cases of breast cancer

tissues has levels above this value (27.5%). This is in agreement with previous reports. Thus, Slamon *et al.* (1987 and 1989), Pauletti *et al.* (2000) and Press *et al.* (2002) who reported that c-erbB2 overexpression was found in 20-30% of breast cancer. Taking 10 HNU/ $\mu$ g as a cut off level, it was found that 8/40 (20%) of breast cancer tissues had levels above this value. This is in agreement with Regidor *et al.* (1995). Moreover, marked variations of c-erbB2 levels were observed with levels varying from 0.8 to 12.43 HNU/ $\mu$ g. This heterogeneity was also reported by Kalogeraki *et al.* (1996).

In the present study the protein levels of c-erbB2 in the breast cancer tissues correlated with stage of disease being significantly higher in advanced stages than early stages (Table 5). Moreover, tissue c-erbB2 protein levels also correlated with number of lymph nodal involvement (Table 6) reflecting tumor burden (Table 7). Tumor burden is a measure of total tumor cell mass. In each cancer lesion measurable by physical examination, chest roentogram and lymphogram, the tumor areas, the tumor areas are calculated as the product of greatest length and breadth of the primary and secondary lesions. The sum of the tumor areas in each patient at a given time was used as an estimation of the total cancer burden (Von Eyben, 1978). Clearly, tumor burden increases with advancing stage of breast cancer and this results in increased c-erbB2 protein levels. In this respect it should be considered that c-erbB2 is able to confer many of the characteristics of a cancerous cell, including uncontrolled proliferation, resistance to apoptosis and increased motility. Over-expression of c-erbB2 leads to cell transformation, a phenomenon largely dependent on increased cell proliferation. The molecular mechanism implies the disruption of cell cycle checkpoints. In particular, regulators of the G<sub>1</sub>/S transition are under the control of c-erbB2-dependent pathways, including the Ras/Erk, p38, MAPK and P13k pathways. Consequently, activation of c-erbB2 promotes the expression of various D-type cyclins and regulates the activity of the p21<sup>cip-WAF-1</sup> and p27 cyclin-dependent kinase (CDK) inhibitors by controlling their expression levels, phosphorylation and nuclear localization (Holbro *et al.*, 2003).

C-erbB2 transforming ability also involves prevention of programmed cell death. Because P13k/ AKt signaling is an important pathway in the content of cell survival, it is often considered the main pathway mediating c-erbB2 receptors antiapoptotic machinery. For instance, Akt mediated phosphorylation prevents association of the proapoptotic Bcl family member BAD with Bcl-XL, allowing Bcl-XL to

promote survival. Similarly, Akt could inhibit several components of the apoptosome, such as caspase-9 or APF-1. Akt also affects pro-apoptotic molecules indirectly, phosphorylation of Forkhead transcriptional regulators prevents their nuclear localization and as a consequence transcription of a number of pro-apoptotic genes (Brunet *et al.*, 2001). Interestingly, c-erbB2 was also shown to prevent tumor necrosis factor-induced apoptosis via the Akt/NF-KB pathway (Zhou *et al.*, 2000). C-erbB2 confers both mitogenic and survival signals. It also regulates breast cancer cell motility in vitro (Spencer *et al.*, 2000) and metastasis in various models (Minn *et al.*, 2005). Cell motility is a complex multi-step process which implicates major morphogenetic events and timely and spatially regulated changes in cell adhesion (Ridley *et al.*, 2003). One of the earliest events consists of formation of membrane extensions, fillopodia and lamellipodia, filled with a dense meshwork of actin filaments. C-erbB2 signaling can influence the molecular mechanisms controlling polymerization of this meshwork and affect focal adhesions via diverse mechanisms (Feldner and Brandt, 2002 and Benlimane *et al.*, 2005). Recently a novel effector of c-erbB2, Memo (mediator of c-erbB2-driven motility), was shown to be involved in c-erbB2-dependent migration of breast tumor cells (Marone *et al.*, 2004). Data indicate that Memo controls microtubule overgrowth toward cell cortex (Badache and Goncalves, 2006). Finally, c-erbB2 also controls cell migration indirectly via regulated expression of mitogenic genes at the translational (Tan *et al.*, 2005) or transcriptional levels (Vial *et al.*, 2003). Thus c-erbB2 regulates cell motility through an intricate network of signaling pathways, the complexity of which is only emerging. The observation that c-erbB2 cooperates with signaling emanating from factors such as hepatocyte growth factor (Khoury *et al.*, 2005) and TGF- $\beta$  (Muraoka *et al.*, 2003) to promote an invasive phenotype adds yet another layer of intricacy to the picture (Badache and Goncalves, 2006). Since c-erbB2 is also known to promote expression of pro-invasive proteases (Mazumdar *et al.*, 2001) and pro-angiogenic factors (Kumar and Yarmand-Bagheri, 2001), it appears that c-erbB2 can contribute several distinct capabilities required to complete tumorigenesis (Hanahan and Weinberg, 2000). This explains with increased tumor aggressiveness, increased rates of recurrence and increased mortality (Goldhirsch *et al.*, 2005). Therefore, the correlation between high tumor burden, increased number of nodal metastasis and advanced stage. As evident in Table (9), increased c-erbB2 levels reflected prognosis in the form of 4 years free survival. This in agreement with reports of Albanell



*et al.* (1996), Andrulis *et al.* (1998), Harris *et al.* (2001) Ali *et al.* (2002) and Classen *et al.* (2002).

When the pathological type of the tumor was considered, it was found that c-erbB2 protein levels were significantly higher in infiltrating ductal carcinoma compared with other pathological varieties (Table 8). This in agreement with Bose *et al.* (1996), Mack *et al.* (1997) and Rosenthal *et al.* (2002). Thus, c-erbB2 amplification and protein overexpression have been associated consistently with high tumor grade, DNA aneuploidy, high cell proliferation, negative assays for nuclear protein receptors for estrogen and progesterone, p53 mutation, topoisomerase II-a amplification, and alterations in a variety of other molecular markers of breast cancer invasiveness and metastasis (Stark *et al.*, 2000, Navolanic *et al.*, 2003 and Zemzoum *et al.*, 2003).

Since previous data have suggested that c-erbB2 could have a causal role in tumor formation, it was anticipated that inactivating c-erbB2 might impede cancer progression in those patients with overexpressed c-erbB2. Recent clinical reports, showing that indeed a humanized antibody targeting c-erbB2, trastuzumab (Herceptin) which specifically bind the extracellular domain can dramatically improve outcomes of women with c-erbB2 expressing tumors (Badache and Goncalves, 2006).

In the present study, the p53 protein levels were also determined by ELISA in cytosols of healthy breast tissues, benign breast diseases and breast cancer. The mean levels in benign breast diseases tissues were significantly higher than healthy tissues (Table 2 and Fig. (2). Millikan *et al.* (1995) reported that 23.3% of patients with benign breast diseases that exhibit atypical epithelial proliferation or fibroadenoma that may be at increased risk of invasive cancer showed focal or diffuse p53 protein immunoreactivity. Point mutations were found in 8.3% of cases. Only 2/5 of patients with p53 mutations, showed p53 overexpression. Lizard-Nacol *et al.* (1995) analyzed p53 mutations in a group of benign breast diseases including fibroadenomas, benign phyllode tumors and fibrocystic diseases. Of these 23% had p53 mutations. Younes *et al.* (1995) reported elevated p53 accumulation in benign breast lesions, of these 30% of fibroadenomas, non premalignant breast lesion were positive. By long term follow up showed that 12% of patients with p53 accumulation and 7% of negative cases, developed breast carcinoma. It seems likely that p53 immunoreactivity in breast lesions should not be used as exclusive evidence of malignancy and may not necessarily identify a subset of patients with benign breast diseases at an increased

risk of breast cancer. In the study of Wells *et al.* (1995), p53 protein levels in patients with benign breast diseases associated with apocrine adenosis had occasional p53 protein positive cells, in 28.6% Zhang *et al.* (1997) reported that in sporadic breast cancer with a history of benign breast diseases, p53 point mutation were observed in 25% of case. Lisboa *et al.* (1997) reported that 7.6% of patients with benign breast diseases (1/13) showed p53 gene abbreviations, this case is a case of atypical ductal hyperplasia. According to these authors p53 mutations occur early in carcinogenesis, as mutations were detected in atypical ductal hyperplasia. Rao *et al.* (1997) reported that 8% of their cases of benign breast diseases showed p53 positivity. Gulaugsdottir *et al.* (2000) found p53 mutations in some benign breast diseases who did not progress to breast cancer. According to these investigators the clinical significance of p53 mutations in benign breast diseases remains to be determined. Kandel *et al.* (2000) demonstrated that p53 protein accumulation detected in normal or benign breast diseases tissues was associated with a 2.5 fold increase in the risk of subsequent breast cancer.

On the contrary, Moriki *et al.* (1995) reported that non of the benign breast diseases expressed p53 protein. Also, Alexiev *et al.* (1997) reported that non of their benign breast diseases showed p53 positivity.

In breast cancer tissues, p53 protein levels were significantly higher than the corresponding levels in healthy and benign breast diseases tissues (Table 2 and Fig. 2). This agrees with the reports of Qi *et al.* (1994) and Kalogeraki *et al.* (2000). Patients with advanced stage, having higher number of lymph node metastasis, high tumor burden, infiltrating ductal carcinomas and postmenopausal women showed significantly higher levels (Table 4, 5, 6, 7 and 8). It has been reported that breast tumors expressing high amount of p53 are more frequently estrogen receptor-negative and progesterone receptor negative. They are also associated with a high proliferation rate, high histological and nuclear grades, aneuploidy and poorer survival (Fehi and Irminger-Finger, 2004). Recent technological advances using multipl RNAs microarray technique or proteins (tissue arrays) in tumor samples or breast cancer cell lines. These studies have revealed that the breast tumors could be sorted into a very few classes characterized by the high level of expression of specific groups of genes/proteins. Moreover, these classes are "stable" as most individual lesions largely maintain there "portrait" when they evolve from in situ to the metastatic state (Lacroix *et al.*, 2004). The number of classes that have been defined in most micro-array

based or tissue array-based studies is three. About two-thirds of tumors express features characteristic of luminal cells. These lesions are often well differentiated, have a low grade and have high levels of cytokeratins and estrogen and progesterone receptors. In contrast to the "luminal-like" lesions, about 20% of tumors have low level of the above cited markers but have high levels of proliferation markers. Most of these basal/myoepithelial-like tumors are poorly differentiated and have a high grade. Finally, tumors over-expressing c-erbB2 as a consequence of gene amplification constitute a third class. It appears that p53 mutation is much more frequent in the basal/myoepithelial-like and c-erbB2 classes than in the "luminal-like" one 82.71 and 31% respectively (Sorlie *et al.*, 2001). Moreover, the most well differentiated tumors have a very low level of p53 alteration, 13% (Sorlie *et al.*, 2001). Of note up to 100% mutant p53 have been observed in medullary carcinoma, a specific subtype of breast cancer with a basal/myoepithelial-like phenotype (de Cremoux *et al.*, 1999). These reports could explain the significantly higher levels of p53 and cerb B-2 in breast cancer tissues of advanced stage, nodal involvement, high tumor burden and ductal carcinomas. Also, they reflect prognostic value of these 2 proteins. This classification affords an explanation of the marked heterogeneity of p53 protein levels observed in the present study (Fig. 2) and in the study of Kalogeraki *et al.* (2000). Nevertheless, based on these reports the significant positive correlation ( $r=0.72$  and  $p<0.001$ ), Table (10) between c-erbB2 and p53 protein levels could be explained. According to Tsutsui *et al.* (2003), p53 protein and cerb B-2 alone have an independent prognostic significance when analyzing the two variables separately. The relative risk for both was 6.37 while the relative risk of p53 alone, c-erbB2 alone and the group positive for one factor ranged between 2.18 and 2.93.

The existence of breast tumor classes suggests that any tumor biology reflect to a large extent the biology of the cell of origin at the time of initiation. Tumor originating from more undifferentiated epithelial cells have a rapid growth pattern and more aggressive behavior and outcome compared with those originating in more differentiated epithelial cells. Thus, neoplastic progression might be p53-dependent in the tumors with a less-differentiated "basal/myoepithelial-like" phenotype and those over-expressing c-erbB2, while it might be p53-independent in those tumors with a more differentiated pure luminal form (Lacroix *et al.*, 2006).

In conclusion, the present study using ELISA technique, a simple technique proved to be correlated with advanced and sophisticated molecular biological techniques, revealed that c-erbB2 and p53 were significantly elevated in breast cancer tissues, reflecting biological behavior of the tumor. The elevated levels correlated with prognosis. However, further studies are needed to further clarify the role of both proteins in tumor initiation and progression especially in benign breast diseases progressing to breast cancer. Meanwhile, due to the number of p53 controlled functions, the diversity of its mutations, the multiplicity of the proteins constituting its "interactome" and the genetic variability inherent to cancer cell progression may result in a tumor suppressor gene may result in a tumor suppressor effect as well as an oncogenic action of p53.

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