

The role of Apoptotic Markers in predicting the response to Neoadjuvant Chemotherapy in Breast Cancer - A prospective clinical study.

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Abstract: Neo-adjuvant chemotherapy is an integral part of multi-modality approach in the management of locally advanced breast cancer and it is vital to predict the response in order to tailor the regime for a patient. The common final pathway in the tumor cell death is believed to be apoptosis or programmed cell death and chemotherapeutic drugs like other DNA-damaging agents act on rapidly multiplying cells including both the tumor and the normal cells by following the same common final pathway. Absence or decreased apoptosis has been found to be associated with chemo resistance. The change in expression of apoptotic markers (Bcl-2 and Bax proteins) brought about by various chemotherapeutic regimens is being used to identify drug resistance in the tumor cells. A prospective clinical study was conducted to assess whether apoptotic markers (Bcl-2/Bax ratio) could serve as reliable predictors of response to neo-adjuvant chemotherapy in patients with locally advanced breast cancer 30 cases of locally advanced breast cancer after complete routine and metastatic work up were subjected to Trucut biopsy and the tissue evaluated immunohistochemically for apoptotic markers (Bcl-2/Bax ratio). Three cycles of Neoadjuvant Chemotherapy using FAC regime (5-fluorouracil, adriamycin, cyclophosphamide) were given at three weekly intervals and patients assessed for clinical response after each cycle. Modified radical mastectomy was performed in all patients three weeks after the last cycle and the specimen were re-evaluated for any change in the Bcl-2/Bax ratio. The immunohistochemical response (change in the Bcl-2/Bax ratio) and the clinical response were correlated and compared. Descriptive studies were performed with SPSS version 10 and the significance of response was assessed using paired t-test. Significance of correlation between various variables was assessed using chi-square test and coefficient of correlation calculated by Pearson correlation coefficient. There was a statistically significant correlation observed between clinical and immunohistochemical response (Bcl-2/Bax ratio) to neoadjuvant chemotherapy. Increase in the ratio (i.e. increase in the expression of Bcl-2) predicted a poor response to neoadjuvant chemotherapy. It was observed in this study that apoptotic markers could reliably predict the response to neoadjuvant chemotherapy in patients with breast cancer. Chemoresistance being an important aspect in tailoring the therapy for a particular patient, the changes in the Bcl/Bax ratio could be utilized in planning an alternative regime.

Key words: *Apoptosis, apoptotic markers, neoadjuvant chemotherapy*

Introduction

Carcinoma of the breast is the leading cause of cancer in women all over the world. It is the second most common malignancy in Indian women after carcinoma of the uterine cervix¹. In the past few years, considerable research has been done on the molecular aspects of breast cancer. The recognition that tumor growth rate is a product of proliferative activity and the rate of cell death has lead to a reappraisal of traditional views of tumor response and resistance to cytotoxic drugs². Apoptosis is a closely regulated form of active cell death defined by characteristic biochemical and morphological criteria. A large number of anti-cancer agents with widely differing modes of action have been demonstrated to induce apoptosis in vitro, suggesting this as a significant final common pathway through which they exert their clinical effect.

The mechanisms that suppress apoptosis may be important in the development of intrinsic and acquired resistance to cytotoxic drugs.³ It was suggested more than 20 years ago that apoptosis might account for much of the spontaneous cell loss, known from kinetic studies, to occur in many tumors. It has been clear for sometime that its extent often is enhanced in tumors by well-established modalities such as chemotherapy, irradiation and hormone ablation. However, during the past few years, advances in the understanding of the control of apoptosis at the molecular

level have extended its potential oncologic significance far beyond the mere provision of a mechanistic explanation for tumor cell deletion. In particular, the discovery that apoptosis can be regulated by the products of certain proto-oncogenes has opened up exciting avenues for future research.⁴ A variety of anti-cancer drugs have been shown to induce extensive apoptosis in rapidly proliferating normal cell population and tumors. Thus enhanced apoptosis is also responsible both for many of the adverse effects of chemotherapy and for tumor regression.⁵ The way in which anticancer drugs induce apoptosis is not known. Better understanding of the processes involved clearly might be expected to lead to improved treatment regimes. However there is an additional important consequence of the realization that anticancer drugs mediate their therapeutic effect by triggering apoptosis³.

Apoptosis is a regulated phenomenon capable of being inhibited and activated. Herein may lay a novel explanation of certain instances of drug resistance. Indeed there is evidence that stimulation of some cells by trophic cytokines or increase in their levels of expression of Bcl-2 proto-oncogeny can greatly increase their resistance to the apoptosis –inducing effect of anticancer drugs. Thus Bcl-2 proto-oncogeny expression may be implicated in the development of resistance of tumors to therapeutic agents and may contribute to tumor growth and perhaps to ontogenesis by allowing the inappropriate survival of cells with DNA abnormalities⁶ Deregulated expression of the Bcl-2 protein represents the best known example of a potent blocker of

apoptosis. Over expression of Bcl-2 has now been shown to protect a wide variety of cell types from induction of apoptosis by many different anticancer agents. Several homologues of Bcl-2 protein have also been shown to act as inhibitors of apoptosis, including Bcl-XL and others as apoptotic proteins including bax.

In vitro data suggests that it is the relative ratios of anti-apoptotic and pro-apoptotic proteins that determine the likelihood of cells to undergo apoptosis in response to chemotherapeutic drugs^{2,7}

“The increasing use of pre-operative chemotherapy (PCT) in breast cancer offers an *in vivo* test bed to test the clinical relevance of these observations.”

It has been studied that low levels of Bax in conjunction with normal Bcl-2 levels might disrupt cellular homeostasis, leading to an accumulation of cells, which might thus become susceptible to secondary mutagenic events resulting in malignant transformation⁸. A number of studies have shown that the clinical stage of tumor at the time of presentation strongly influences the outcome of treatment. Traditional clinical staging requires accurate measurement of tumor size and assessment of axillary lymph node status. The change in the clinical stage after chemotherapy is also of prognostic significance⁹.

The **aim** of this prospective study was to assess:

- 1) The role of apoptotic markers (ratio of Bcl-2 / Bax gene expression) in predicting the response to neoadjuvant chemotherapy.
- 2) The response to neoadjuvant chemotherapy, in terms of tumor size and axillary lymph node status.
- 3) To correlate the clinical and immunohistochemical response to neoadjuvant chemotherapy in carcinoma breast

Methods:

Thirty (30) FNAC proven cases of locally advanced breast carcinoma according to AJCC (American Joint Committee On Cancer) classification were included in the study. Before contemplating the study, approval of the IRB and the Ethical committee of the hospital was taken. A thorough clinical and ultrasonographic examination (USG) of all the patients including the opposite breast was performed to stage the disease accurately. A core biopsy using a tru-cut needle was performed for immunohistochemical estimation of the apoptotic markers i.e. base-line Bcl-2/Bax ratio before initiating the chemotherapy. Routine and metastatic work up was done including complete blood examination (total blood count, platelet count), chest radiograph, ECG (Echocardiography when ECG had a positive finding), liver function tests, Bone Scan, USG abdomen, KFT (Kidney function tests).

Patients were subjected to three cycles of FAC regime (cyclophosphamide 600 mg/m², adriamycin -50 mg/m², 5-fluorouracil-600 mg/m²) at an interval of three weeks. Before each cycle the patient was clinically and sonologically examined for the breast tumor size, axillary lymph node status & appearance of systemic metastasis. All patients were given the same antitoxicity treatment according to a standardized unit protocol including adequate hydration and inject able antiemetics before initiating the chemotherapy.

All patients were subjected to Patey’s modified radical mastectomy three weeks after the last cycle and the specimen were again subjected to immuno-histochemistry to evaluate for any change in the Bcl-2/Bax ratio and for the histological tumor size, margins.

Objective clinical response was defined as >50% reduction in the tumor size after completion of three cycles of NACT, as assessed clinically, sonologically and histologically. Immunohistochemical response was taken as decrease in the Bcl-2/Bax ratio. Any increase or no change in this ratio was considered as no response.

Immuno-histochemical methods : Biopsy specimen was preserved in buffered formalin solution. Five-micron sections were prepared from paraffin embedded blocks on poly-l-lysine coated glass slides. Sections were deparaffinized in xylene for 15 min. and hydrated in alcohol for 15 minutes. Further, incubation was done in 0.3% hydrogen peroxide in methanol solution for 45 min. The slides were washed with citrate buffer and kept in a water bath at 90–95°C for 45 min. for antigen retrieval. Sections were washed with Tris buffer saline (TBS) solution and incubated with blocking antibodies (DAKO monoclonal mouse antihuman Bcl-2 oncoprotein for Bcl-2 expression and polyclonal rabbit antihuman for Bax expression) at 37°C. Sections were washed with TBS solution. Incubation with avidin-biotin complex (ABC) was done at 37°C for one hour and washed with TBS solution. 3,3 Diaminobenzidine tetra hydrochloride solution applied for 3–5 min. Counter-staining with haematoxylin solution done for 3–5 min. Sections were washed with distilled water, air dried and mounted using DPX mountant.

For Bax, positive controls were taken as germinal centers of the lymphoid follicles and normal breast tissue and negative control was taken as the test slide without primary antibody. For Bcl-2, positive controls were the mantle zone of lymphoid follicles and the negative controls were the test slides without primary antibody.

a) *The pattern of positive staining for Bcl-2 and Bax was cytoplasmic, granular. b) The primary antibodies for Bcl-2 and Bax were procured from DAKO. c) Bax-Rabbit Anti-Human code no. A 3533. d) Bcl-2 Monoclonal Mouse Anti-Human code no. M 0887. e) Dilution for both was 1: 40.*

The results were interpreted on the basis of two criteria:

- (1) Percentage of cells showing immune bodies; <5%: score = 0, 5–25%: score = 1, 25–75%: score = 2, >75%: score = 3; (2) Intensity of staining; mild: score = 1, moderate: score = 2, intense: score = 3.[Fig.1, Fig.2, Fig.3, Fig.4]

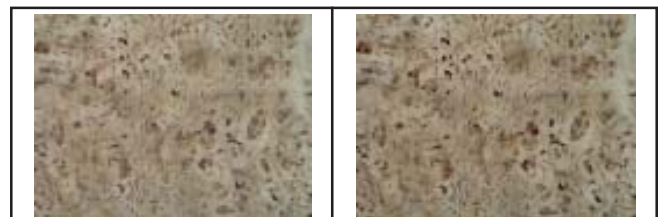


Figure.1: Immunohistochemistry showing positive (moderate) staining of tumor cells with Bax antibody

Figure.2: Immunohistochemistry showing positive (intense) staining with Bax antibody



Figure.3: Immunohistochemistry showing moderate cytoplasmic staining of tumor cells with Bcl-2 antibody

Figure.4: Immunohistochemistry showing positive (intense) cytoplasmic staining of tumor cells with Bcl-2 antibody.

Table 1 Cohort Distribution (n=30)

		Clinical		Immuno-histochemical	
		Response	No response	Response	No response
Menopausal status					
Pre-menopausal	53.3%	62.5%	37.5%	56.2%	43.8%
Post-menopausal	46.7%	71.4%	28.6%	64.2%	35.8%
Tumor size					
<5cm	0%	—	—	—	—
5-8cm	56.6%	68.7%	31.3%	60%	40%
8-10cm>	26.6%	79%	29%	60%	40%
10cm	16.6%	66.6%	33.3%	60%	40%
Axillary LN status					
N0	0%	—	—	—	—
N1	57.3%	—	—	—	—
N2	42.7%	—	—	—	—
Total		70%	30%	60%	40%

"Since there was a strong correlation between the intensity of staining and percentage of cells showing immune bodies, the percentage of cells showing immune antibodies alone was considered for calculating the Bcl-2/Bax ratio".

Statistics : Descriptive studies were performed with SPSS version 10. The significance of response assessed using paired t-test. Significance of correlation between various variables assessed using chi-square test and coefficient of correlation was calculated by Pearson correlation coefficient. The clinical and immunohistochemical response were then correlated and compared.

Results:

Age of the patients ranged from 28-71 years with the mean age of 45.5 years and a standard deviation of 11.732 yrs; the median and mode were 43 years and 40 years, respectively.

Comparison of tumor size before and after neo adjuvant chemotherapy was performed using paired 't' test ,at the

Table 2. Immunohistochemical response following neoadjuvant chemotherapy

Total number of patients = 30

Immunohistochemical Response	No. of patients	% Of patients
Responders	18	60%
Nonresponders	12	40%

Table 3. Comparison between immunohistochemical and clinical response

		Immunohistochemical Response.			
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Non responders	12	40.0	40.0	40.0
	Responders	18	60.0	60.0	100.0
	Total	30	100.0	100.0	

confidence limit of 95% (P <0.05) chi-square test was applied and the clinical response to NACT in terms of the change in the tumor size was found to be statistically significant.

Response observed in the axillary lymph nodes: Out of total of 17 patients that were N1 before NACT 5 (29.4%) were Down-staged to N0 whereas 12 (70.6%) remained N1. Out of a total of 13 patients who were N2 before NACT; 1 (7.7%) was down staged to N0 and 6 (46.2%) were downstaged to N1 whereas 6 (46.2%) remained N2. With confidence limit of 95% (P0.05%) chi-square test was applied and the response to NACT in terms of the down-staging of the axillary lymph node status

Clinical Response

Valid		Frequency	Percent	Valid Percent	Cumulative Percent
		Non responders	9	30.0	30.0
	Responders	21	70.0	70.0	100.0
	Total	30	100.0	100.0	

Clinical. Response. * Immunochemical Response. Cross tabulation

di.Res.		Count	Immunohist. Response.		Total
			Nn responders	Res-ponders	
Non responders	Count	9		9	
	% within cli. Res.	100.0%		100.0%	
Responders	Count	3	18	21	
	% within cli. Res.	14.3%	85.7%	100.0%	
Total	Count	12	18	30	
	% within cli. Res.	40.0%	60.0%	100.0%	
	% within imm. Res.	100.0%	100.0%		

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	19.286(b)	1	.000		
Continuity Correction(a)	15.880	1	.000		
Likelihood Ratio	23.156	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	18.643	1	.000		
N of Valid Cases	30				

At 99% confidence limit and degree of freedom =1, n=30; c2 distribution test was applied to the above table and a statistically significant correlation was observed between the clinical and immuohistochemical response.

was found to be statistically significant

Out of the total 30 patients studied 18 (60%) showed a decrease in the Bcl-2/Bax ratio following neoadjuvant chemotherapy. In 9 (30%) of the patients the ratio increased where as in 3 (10%) of the patients the ratio remained unchanged. Paired 't' test was applied to find the statistical significance and immunohistochemical response in terms of change in ratio of bcl-2/bax gene expression was found to be statistically significant (Table 2 & 3)

Acute vomiting (taken as minimum of three vomiting in 24 hours) was observed in 63.3% patients. 81% clinical responders

Table 4 : Toxicity and Response (n=30)

	Total N=30	Clinical responders	Clinical non-responders	Immuno-histochemical responders	Immuno-histochemical non-responders
Acute vomiting	63.3%	81%(p=0.002)	22.2%	78%(p=0.04)	41.7%
Alopecia	60%	86%(p=0.000)	0%	94%(p=0.000)	8.3%
Leucopenia	10%	14%(NS)	0%	17%(NS)	0%
Alopecia+Acute vomiting	46.7%	67%(p=0.001)	0%	72%(p=0.001)	8.3%

had vomiting (p=0.002) and 78% immunohistochemical responders also had vomiting (p=0.04) which was statistically significant. Alopecia (taken as complete alopecia) was observed in 86% clinical responders (p=0.000) and 94% immuno-histochemical responders (p=0.000), which was also significant. Leucopenia (using the WHO criteria) was observed in only 14% and 17% of clinical and immuno-histochemical responders respectively and was found to be an insignificant observation factor in our study. When multiple toxicities were correlated with the clinical and immuno-histochemical response, 46.7% of patients had both acute vomiting and alopecia. 67% clinical responders (p=0.001)

had both vomiting and alopecia. 72% immunohistochemical responders ($p=0.001$) had both vomiting and alopecia. A positive significant correlation was found between the presence of vomiting ($r=+0.558$), alopecia ($r=+0.802$) and response to neoadjuvant chemotherapy. A significant negative correlation was also observed between the absence of side effects and poor response to neoadjuvant chemotherapy. The toxicity observed correlated significantly with the response to neoadjuvant chemotherapy i.e. responders showed significant toxicity also (Table 4).

Discussion:

Carcinoma of the breast is the leading cause of cancer in women all over the world and the second most common malignancy in India after carcinoma of the uterine cervix¹. No other common epithelial cancer has been so carefully studied and so extensively characterized biologically^{1,2}. In developing countries like India rate of locally advanced breast cancer at first diagnosis is estimated to be as high as 25%–30%^{2,5}. The treatment of locally advanced breast carcinoma (LABC) has also evolved from primarily local modalities to treatment regimens that combine both systemic and local therapy. The realization that patients with LABC are likely to have undetectable micro metastases at diagnosis has led to systemic treatment assuming major focus of the multi-modality approach as the studies have confirmed that surgery alone is an inadequate treatment in the management of these patients. Even aggressive surgical techniques have been observed to have a higher incidence of local recurrence in these patients^{10,11}. Most importantly surgery does not change the pattern of distant failure in patients who probably have micrometastatic disease at the time of diagnosis^{10,13}. Multi-modality therapy that included surgery, radiation therapy, chemotherapy, hormonal therapy has had the greatest impact on survival in patients with LABC^{10,13}.

Neoadjuvant chemotherapy (NACT) : A new approach in the form of neoadjuvant chemotherapy was first reported in the 1970s and was initially utilized to convert unresectable tumors to smaller tumors making them more amenable to local control with either surgery or radiotherapy. An added advantage of this approach was the ability to assess patient's response to treatment both clinically after a defined number of courses of chemotherapy and pathologically after surgical resection. Perez and colleagues reported their results of a pilot study by the South-Eastern Cancer Study Group in 1979 that the primary tumor showed partial regression (>50%) in 65% of patients after two courses of FAC¹⁶. NACT has also shown benefits in the operable breast cancers by increasing the chances of breast conservation by up to 90% in some trials^{10,13}. The other important advantage of NACT is that it represents an *in vivo* chemo sensitivity test for assessment of tumor response from which prognostic information can be obtained. It provides an early treatment of the micrometastatic disease, counteracting the increased growth rate possibly determined by the shrinkage of the tumor. The down staging converts an inoperable case amenable to curative resection^{10,13}.

Apoptosis : Introduced by Kerr et al (1972), to describe characteristic morphological changes seen during programmed cell death³. It is defined as a closely regulated form of active cell death defined by characteristic biochemical and morphological criteria^{3,14,15}. A wide range of anticancer drugs with widely differing modes of action have been demonstrated to induce

apoptosis *in vitro*, suggesting this as a significant common final pathway through which they exert their clinical effect. Further more the mechanisms that suppress apoptosis may be important in the development of acquired resistance to cytotoxic drugs. Apoptosis or programmed cell death plays an important role in the regulation of tissue development, differentiation and homeostasis. It is therefore possible that deregulation of apoptosis contributes to the pathogenesis of cancer^{3,13-15}. Apoptosis can be differentiated biochemically and morphologically from necrosis by the following criteria¹⁶:

- (1) Chromophin condensation;
- (2) Membrane blebbing;
- (3) Appearance of apoptotic bodies;
- (4) Fragmentation of genomic DNA

Certain biochemical and genetic events have been identified that are associated with multiple cell types including mammary epithelium. These include the DNA fragmentation via end nuclease activation and cleavage of intracellular proteins, expression of bcl-2 family members, tumor suppressor gene p-53 directed events, proto-oncogene activation and activation of transmembrane receptor signaling pathways such as tumour necrosis factor^{4,17-22}. Although little is known about the mechanisms, which regulate apoptosis in epithelial cells, it is conceivable that defects in apoptosis related genes are involved in the pathogenesis of human cancers. The hypothesis is supported by the fact that the tumor suppressor gene product p-53, which is frequently mutated or deleted in breast cancer, is involved in regulating apoptosis²³. The heterogeneous nature of breast cancer has resulted in overwhelming interest in search for prognostic markers to identify patients who might benefit most from the therapeutic modalities available.

Assessment of apoptosis and individual components of apoptotic pathway are therefore relevant in determining prognosis in a particular patient²⁴. DNA damaging agents such as ionizing radiations and chemotherapeutic drugs also induce apoptosis. Sakakura et al have shown an association between increased resistance to chemotherapeutic agents and decreased capacity to undergo apoptosis²⁵. Central to this response are proteins that modulate apoptosis, including Bcl-2 and Bax gene products. Bcl-2 is anti-apoptotic in function, whereas Bax is proapoptotic and it is the interaction between the two that determines the likelihood of a tumor to undergo cytotoxic drug mediated regression. Therefore any increase in Bcl-2 or decrease in Bax will push the balance towards chemo resistance and an increase in Bax or decrease in Bcl-2 will result in increased apoptosis²⁶⁻³⁰. It was observed in a study conducted by Kymionis et al¹⁵, that increase in the ratio of anti apoptotic protein Bcl-2 to pro-apoptotic protein i.e. Bax results in markedly enhanced resistance of tumor cell lines to the cytotoxic effects of essentially all currently available chemotherapeutic drugs.

In the present study the clinical response in terms of reduction in tumor size correlated significantly with the immunohistochemical response in terms of change in the Bcl-2/Bax ratio.

Conclusion:

Neoadjuvant chemotherapy is routinely used in the management of locally advanced breast cancer. The present study highlights the importance of utilizing apoptotic markers in predicting the response to neoadjuvant chemotherapy so that an alternate regime could be planned in non-responders.

References:

1. Paymaster JC: Cancer of the breast in Indian Women. *Surgery* 1956, 40:372-376.
2. Ellis PA, Smith IE, Detre S et al: Reduced apoptosis and proliferation and increased Bcl-2 in residual breast cancer following preoperative chemotherapy. *Breast Cancer Research and Treatment* 1998, 48: 107-116.
3. Kerr JF, Winterford CM, Hormon BV: Apoptosis: its significance in cancer and cancer therapy. *Cancer* 73 (8): 2013 - 2026
4. Hahm HA, Davidson NE: Apoptosis in the mammary gland and breast cancer. *Endocrine Related Cancer* 1998, 5:199-211.
5. Jussawala DJ, Yeole BB, Natekar MV, Narayan RA: Epidemiology of breast cancer in India. *Indian J Cancer* 1978,12:231-242. INCLUDEPICTURE "http://www.biomedcentral.com/sfx_links.asp?getImage" * MERGEFORMATINET
6. Frassoldati A, Adami F, Banzi C, et al: Changes of biological features in breast cancer cells determined by primary chemotherapy. *Breast Cancer Res And Treat* 1997, 44: 185 - 192.
7. Oltvai ZN, Milliman CL, Korsmeyer SJ: Bcl-2 heterodermize in vivo with a conserved homologue, bax, that accelerates programmed cell death. *Cell* 1993, 74: 609 - 615.
8. Hockenbery DM, Nunez G, Milliman : Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990, 348: 334 - 336.
9. Bargou RC, Daniel PT, Mapara MY, et al: Expression of the bcl-2 gene family in normal and malignant breast tissue: low bax expression in tumor cells correlates with resistance towards apoptosis. *Int J Cancer* 1995, 60:844-859. INCLUDEPICTURE "http://www.biomedcentral.com/sfx_links.asp?getImage" * MERGEFORMATINET
10. Eltahir A, Heys SD, Hutcheon AW, et al: Treatment of large and locally advanced breast cancer using neoadjuvant chemotherapy. *Am J Surg* 1996, 175: 127 - 132.
11. Bonadonna G, Valagussa P, Zambetti M: Locally advanced breast cancer: 10 year results after combined treatment. *Proceed Am Soc Clin Oncol* 1988, 7: 9. -INCLUDEPICTURE "http://www.biomedcentral.com/sfx_links.asp?getImage" * MERGEFORMATINET
12. Buzdar A, Singletary S, Booser D: Combined modality treatment for stage III and inflammatory carcinoma of the breast. M.D. Anderson Cancer Center experience. *Surg Oncol Clin North Am* 1995, 4: 715. -INCLUDEPICTURE "http://www.biomedcentral.com/sfx_links.asp?getImage" * MERGEFORMATINET
13. Singh G, Singh DP, Gupta D: Neoadjuvant chemotherapy in locally advanced breast cancer. *J Surg Oncol* 1996, 61:38-41.
14. Blomqvist C, Elomma I, Rissanen P: Influence of treatment schedule on toxicity and efficacy of cyclophosphamide, epirubicin and fluoracil in metastatic breast cancer-a randomized trial comparing weekly and every four week administration. *J Clin Oncol* 1993, 11:467-473.
15. Kymionis GD, Dimitrakakis CE: Can expression of apoptosis genes, bcl-2 and bax predict survival and responsiveness to chemotherapy in node negative breast cancer patients? *J Surg Res* 2001, 99: 161 - 168.
16. Searle J, Lawson TA, Abbott PJ: An electron microscope study of the mode of cell death induced by cancer chemotherapeutic agents in populations of proliferating normal and neoplastic cells. *J Pathol* 1975, 116: 129 - 138.
17. Nabusada Shinoura, Yoko Yoshida, Miyako Nishimura, et al: Expression of BCL-2 Determines Anti- or Proapoptotic Function. *Cancer Research* 59: 4119 - 4128. - INCLUDEPICTURE "http://www.biomedcentral.com/sfx_links.asp?getImage" * MERGEFORMATINET
18. Perez P, Presant C, Philpott G: Phase I-II study of concurrent and multi drug chemotherapy in advanced carcinoma of breast: a pilot study by the south-eastern cancer study group. *Int J Radiat Oncol Biol Phys* 1979, 5: 1329.
19. Fisher B, Brown A, Mamounas E: Effect of preoperative chemotherapy of loco regional disease in women with operable cancer-Findings from National Surgical Adjuvant Breast and Bowel Project B-18. *J Clin Oncol* 1997, 15: 2483 - 2493.
20. El-Didi MH, Moneer MM, Khaled HM: Pathological assessment of response of locally advanced breast cancer to neo adjuvant chemotherapy and its implication for surgical management. *Surg Today* 2000, 30: 249 - 254.
21. Diadone MG, Silverstrini R, Luisi A: Changes in biological markers after primary chemotherapy in breast cancers. *Int J Cancer* 1995, 61: 301 - 305.
22. Mamounas EP, Fisher B: Preoperative (neoadjuvant) chemotherapy in patients with breast cancer. *Semin Oncol* 2001, 28: 389 - 399.
23. Thor AD, Reed JC, Sato T: Immunohistochemical analysis of bax and bcl-2 in p53 immunosuppressive breast cancers. *US Canad Acad PatholAbst* 1996, 26 A: 132 - 135. - INCLUDEPICTURE "http://www.biomedcentral.com/sfx_links.asp?getImage" * MERGEFORMATINET
24. Miyashita T, Reed JC: Bcl-2 gene transfer increases relative resistance of 549.1 and WEH 17.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. *Cancer Res* 1992, 52: 5407 - 5410.
25. Wyllie AH, Kerr JFR, Curie AR: Cell death-the significance of apoptosis. *Internat Rev Cytol* 1980, 68:251-304. - INCLUDEPICTURE "http://www.biomedcentral.com/sfx_links.asp?getImage" * MERGEFORMATINET
26. Sakakura C, Sweeny EA, Shirahama: Over-expression of bax sensitizes human breast cancer MCF-7 to radiation induced apoptosis. *Int J Cancer* 1996, 67: 101-105.
27. Krajewski S, Krajewska M, Shabaik A: Immunohistochemical determination of bax a dominant inhibitor of bcl-2. *Amer J Pathol* 1994, 145: 1323 - 1336. -INCLUDEPICTURE "http://www.biomedcentral.com/sfx_links.asp?getImage" * MERGEFORMATINET
28. Feuerhake F, Sigg W, Hofter EA: Cell proliferation, apoptosis and expression of bcl-2 and bax in non lactating human breast epithelium in relation to the menstrual cycle and reproductive history. *Breast Cancer Res Treat* 2003, 77: 37 -48.
29. Rasbridge SA, Gillett CE, Seymour AM: The effect of chemotherapy on morphology, cellular proliferation, apoptosis oncoprotein expression in primary breast carcinoma. *Br J Cancer* 1994, 70: 335 - 341
30. Kuerer HM, Newman LA, Smith TL: Clinical course of breast cancer patients with complete pathologic primary tumor and axillary lymph node response to doxyrubicin based neoadjuvant chemotherapy. *J Clin Oncol* 1999, 17: 460 - 469.