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Original Article

EXPRESSION OF THE G2M CHECKPOINT REGULATOR CYCLIN B1 IN BREAST CANCER AND ITS CORRELATION WITH PROGNOSTIC PARAMETERS

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ABSTRACT

Background: Breast cancer is the most common malignancy in Egyptian females. A wealth of literature is available on various diagnostic and prognostic parameters including clinical, pathological and biological factors. The present study focuses on markers of cell cycle proteins as cyclin B1 and parameters of cell cycle kinetics, related growth factors and tissue polyamines in relation to hormone receptor status.

Patients and methods: A total of 102 females made the subject of the study, 72 females with breast cancer, 14 with benign lesions and 16 healthy subjects serving as controls for serum parameters. Normal breast tissue served as control for breast cancer tissues. Tissue polyamines were measured by HPLC. Cyclin B1 was measured by Flow cytometry (FCM) and Western Blotting (WB). Insulin-like Growth factor 1(IGF1) was measured by enzyme immunoassay (EIA). Tumor markers (CEA and CA 15.3), estradiol and progesterone levels were measured by microparticle enzyme immune assay (MEIA).

Results: Cyclin B1 was detected in 17/34 (50%) of the cases tested by FCM and only in 10% of the cases evaluated by WB. Cyclin B1 positivity tended to be associated with high grade tumors. The mean percentage of cyclin B1 positivity was higher in aneuploid tumors than in diploid ones, as well as in tumors with ≥ 10% S-phase fraction (SPF) compared to those with < 10 % SPF. Cyclin B1 positivity was significantly associated with patients of high G2/M phase (p=0.05). Cell cycle analysis showed that ER-ve tumors were significantly associated with high SPF. Tissues polyamines showed significantly increased levels when compared to both benign and normal groups (p< 0.001). Polyamines showed a significant association with ER status (p= 0.04), tumor size (p=0.04) and histopathologic type (p= 0.05). Serum IGF 1 showed insignificant increased level in breast cancer patients less than 45 yrs when compared to the age matched control group. Serum estrogen level showed no change in the breast cancer patients ≥ 45yrs compared to age matched control group, while progesterone level was significantly lower (p< 0.02) in breast cancer patients less than 45yrs old compared to age matched benign and control groups. Serum CEA and CA 15.3 showed significant increase in the breast cancer patients < 45yrs old compared to age matched benign and control groups (p=0.03 and 0.06, respectively).

Conclusion: Polyamines proved value as markers of proliferative activity of breast cancer but their testing needs further standardization. Cyclin B1 is associated with aggressive tumors. Its assessment by FCM proved superior to western blotting. IGF1 might be better used to assess tumor recurrence and monitor cancer patients, and its use needs further evaluation. This study may contribute to understanding breast cancer cell growth biology in Egyptian females in view of improving therapeutic and chemopreventive strategies.

Key Words: Cyclin B1, polyamines, IGF1, tumor markers, breast cancer.

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INTRODUCTION

Worldwide, breast cancer represents a major health problem, being responsible for 20% of annual cancer deaths. In Egypt, data reported indicated that breast cancer ranked as number one (35.1%) among females. Despite progress achieved in screening and management of breast cancer including adjuvant treatment, established prognostic factors proved insufficient to help predicting the prognosis[1]. Breast cancer patients with the same stage of disease can have markedly different treatment responses and overall outcome[2]. Hence, more understanding of breast cancer biology will pave the way for further therapeutic targets based on their critical role in tumorigenesis.[3]

Altered regulation of the cell cycle is a hallmark of human cancers[4]. Cell cycle progression is governed by a series of cyclins and cyclin dependent kinases (CDKs).

Of the various cyclin/CDK complexes involved in cell cycle regulation, cyclin B1/p34cdc2 is of particular interest because it controls G2M phase checkpoint, which in turn, is essential for cell as proliferation. Expression of cyclin B1 has been reported in breast cancer patients[5]. One mechanism suggested for cyclin B1 regulation in MCF-7 breast cancer cell lines was through alteration in its transcription mediated by estrogen[6]. Several pathways have been proposed for estrogenic pleiotropic growth stimulatory effects on breast epithelial cells direct gene regulatory effects mediated by estrogen receptors (ER) resulting in expression of cmyc leading to DNA synthesis and cell division[7] and stimulatory effects on the production of growth factors, particularly epidermal growth factor and IGF1.[8]

The regulation of polyamines level during cell cycle and the biosynthesis of their disruption associated with cell cycle arrest raises the possibility that polyamines pathway might intersect with proteins that regulates cell cycle[9]. Moreover, Polyamine pathway is interlinked with estrogenic regulation of cell growth in breast cancer cells. They are capable of altering the structural organization and DNA binding of estrogen and other steroid receptors[10,11]. Studies demonstrating estrogenic actions on target tissues showed that estrogen stimulates ornithine decarboxylase (ODC) activity, a key enzyme in the biosynthesis of polyamines[12]. Polyamines can also exercise a permissive role in the cooperative effect of estrogen and IGF1 on human breast cancer cell growth[13] and could be critical to the expression of c-myc[14], increased ODC activity, associated with increased risk of recurrence and death.[12]

Up to now, there are only few studies concerning cyclin B1 in clinical breast cancer. Also, studies of this G2M cyclin in clinical breast cancer were mostly done by immunohistochemistry only. Cyclin B1 could be possibly of clinical value once related to other biochemical and clinicopathological factors. Reports on aberrant expression of this cyclin in breast cancer cell line showed controversy.[15]

The aim of this study is to assess the expression of cyclin B1 and polyamines in breast cancer patients as interrelated regulatory components of cell cycle kinetics and their relation to other established prognostic factors.

PATIENTS AND METHODS

The present study included 102 female subjects presented to outpatient clinics of NCI, Cairo University, during the period 2001-2003. They were 71 patients with localized breast tumors, one with recurrent invasive duct carcinoma, 14 with benign breast lesions. Adjacent normal breast tissues from breast cancer patients served as control.

All breast cancer patients were histopathologically diagnosed and clinically staged according to TNM classification of the WHO. Hormone receptor status was determined immunohistochemically. Routine laboratory investigations were done to all patients.

Tissue parameters:

Fresh tissue biopsies were obtained from benign and malignant breast masses. Samples were selected carefully from areas within tumor margins, washed by ice-cold saline and cut into three small pieces. Two of them were kept immediately at -80ºC until further processing for analysis of polyamines and cyclin B1by WB. The third part was chilled on a Petri dish mounted on ice and immediately subjected to mechanical cell disruption in RPMI media to obtain cell suspension required for studying cell cycle kinetics and cyclin B1 by FCM. Ehrlish-ascites carcinoma and hepatoma cell line (HebG2) were kindly supplied by Biology Department, NCI, Cairo University and included in the study as comparative tool in cell cycle studies and cyclin B1 measurement.

Determination of tissue polyamine by HPLC:

Weighed aliquots of control, benign and malignant tissue samples were homogenized in 5-10 volumes of cold 0.3 M perchloric acid (PCA) in the presence of a known amount of internal standard, 1,7 heptanediamine (100 nmol). Clear supernatants obtained after centrifugation of PCA-treated samples were stored frozen (-20ºC) until further analysis. Precolumn derivatization was applied using benzoyl chloride as previously described[16]. Separation was performed using two ChromSep HPLC columns (100mm X 3 mm ID) packed with ChromSphere C18 reversed-phase material (5-µm particles). Volumes of 2-10µl of the solution of derivatized compounds in methanol were injected into the column. The mobile phase was methanol-water. Carbon-pretreated deionized water, filtered over an organic TM cartridge to remove organic contaminants and de-aerated with helium gas, was used for preparation of the mobile phase. Chromatographic analyses were performed on Shimadzu system, USA.

Flow cytometric analysis of cell cycle and cyclin B1 expression in breast cancer tissues:

Mechanical cell disruption:

Part of fresh tumors was weighed, put in Petri dish containing 5ml RPMI 1640 as a nourishment medium (Gibco, New York) and by using forceps and a surgical blade, tissues were gently scraped until the solution became turbid. The solution was then filtered through 80 µm nylon mesh with mesh opening = 40µm (Engineering Findings, Miami, USA) into a 15-ml conical centrifuge tube and centrifuged at 900 rpm for 3min.[17]

Tumor ascites carcinoma cells and HebG2 cell line were treated exactly as tissues except that no mechanical cell disruption was needed. Cell suspensions in PBS were counted, Cell viability was performed using 10 ul trypan blue dye and 10ul of cell suspension. Cell suspensions were fixed in cold 80% ethanol (Sigma, Germany) and maintained at -20ºC to -40ºC for a minimum of two hours. Cells were then centrifuged, rinsed once in PBS, and the cell pellets suspended in 1ml of 0.25% solution of Triton X-100 (Fischer, UK) and kept on ice for five min. Cells were then washed in 5ml PBS and centrifuged[18]. The cell pellets were then suspended in 100ul of PBS and 20ul of anti-cyclin B1 FITC conjugated antibody (Pharmingen, USA) was added and incubated at 4ºC overnight. FITC-conjugated mouse Ig G1 was used as isotype control. Cells were rinsed again with PBS and cell pellets resuspended in a solution containing 10ug/ml propidium Iodide (PI) (Sigma, Germany) and 1ml of 1% RNase A in PBS (Sigma, Germany). The solution was incubated in the dark at room temperature for20 min before measurement. Control cells were prepared as described except that the isotype-specific Ab was used instead of cyclin B1 Ab. The threshold discriminating cyclin B1 positive cells was based on the mean value of fluorescence of the negative cells. Data acquisition was carried out using XL FCM (Coulter Electronics, Miami, FL) after being standardized. DNA content and cyclin B1 were measured at PI and FITC emission, respectively. The aggregates and debris were excluded. Electronic gates were used to subtract background fluorescence of the FITC-conjugated negative controls and to evaluate the percentage of cyclin B1-positive cells. The fluorescence of at least 10,000 events was collected to generate dual parameter histogram. The peak position of Go/G1 and G2M population and the coefficient of variation (CV) of both diploid and aneuploid peaks were determined (Figure 1). Cell cycle evaluation of the DNA histograms and the CV of each peak shown by FCM were performed by Multicycle software, computer program (Phoenix Flow Systems, CA, USA). The mean fluorescence channel ratio (MFCR) of the cyclin B1-positive gated cells, the synthetic phase fraction (SPF) and the ploidy status were determined.[18]

Detection of cyclin B1 in breast tissue extracts by WB19

Tumor tissues and the apparently normal adjacent breast tissue were homogenized and sonicated in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% aprotinin, 1% sodium deoxycholate, 2mM EDTA, 50 mM sodium fluoride, 20ug/ml phenylmethylsulfonyl fluoride, 100 Um sodium vanadate and 1mM dithiothreitol and then centrifuged at 10.000 g for 30 min at 4ºc12. The positive control cell lines Ehrlish ascites carcinoma and HebG 2 cell pellets were subjected to the same process of cell lysis. Thirty microgram protein from cell lysates was electrophoresed on 10% polyacrylamide gel with a wide range molecular weight marker (Amersham, cat RPN 2107) at 70mA current with circulating constant temperature. Proteins were then transferred onto a nitrocellulose membrane (Amersham, UK) and stained with Ponceau S (Sigma, Germany). Membranes were probed with purified anti-human cyclin B1 monoclonal Ab (Pharmingen, USA) at 1:500 dilutions, followed by incubation with horseradish peroxidase-labeled anti-mouse secondary antibody (Amersham, UK) at 1: 5000 dilutions and detected with enhanced chemiluminescence system (Amersham, Germany) . Signal detection of a band at molecular weight 62 KDa corresponds to lane containing cyclin B1 expressed by HebG2.

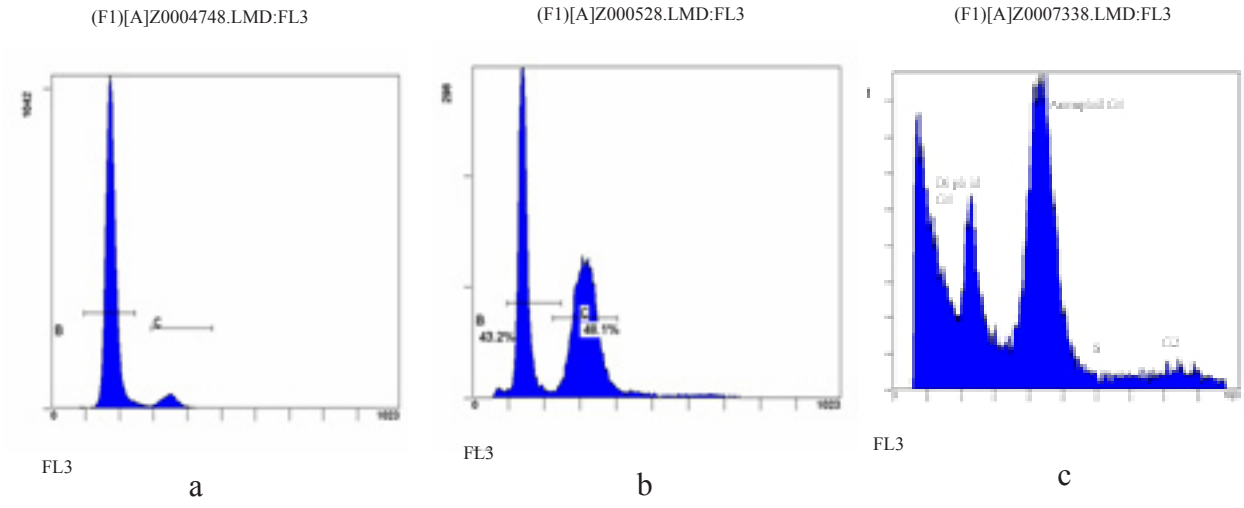


Fig. 1: Flow cytometry DNA histograms of three different breast cancer cases
(a) Diploid pattern with normal Go/G1, S and G2/M phase.
(b) Diploid pattern with 43.2% Go/G1 and 48.1% G2/M populations.
(c) hyperdiploid pattern.

Determination of serum IGF1 was done using ELISA Kit (Biosource, Europe S.A., Belgium). Serum CEA and CA 15.3, estradiol were determined by MEIA technology (Abbott Laboratories Diagnostics, IL, USA). Serum progesterone was done by the Electrochemiluminescence Immunoassay (ECLA) (Roche Elecsys instrument, USA).

Statistical Analysis

Data were analyzed using SPSS statistical package version 11[20]. Numerical data were expressed as mean ± SD, median, minimum and maximum. Qualitative data were expressed as frequency and percentage. For comparison between the three groups for numerical variables, Kruskall-Wallis test was used. Mann-Whitney test was applied for comparison between two qualitative groups. For comparison of qualitative data, Chi-square test was used. Spearman correlation test was applied to correlate numerical variables.

RESULTS

The study involved 3 groups; group 1 (72 breast cancer patients), group 2 (14 benign breast lesions) and group 3 (16 normal healthy volunteers) serving as control for serum parameters. Patients were grouped according to their age into 2 groups; cases≤ 45 years and those > 45 years. Cancer group cases were significantly older than cases with benign lesions ($p < 0.001$). Histopathological criteria of all cases are presented in table 1.

Table 1: Histopathological types of the benign and breast cancer cases.

Group	No	%
Malignant		
Intraductal carcinoma (IDC)	60	83.3
Intralobular carcinoma (ILC)	7	9.7
Non-invasive	2	2.8
IDLC	3	4.2
Total	72	100.0
Benign		
Fibroadenoma	7	50%
Fibrocystic disease	3	21.44
Lipoma	1	7.14
Duct ectasia with mastitis	1	7.14
Fat necrosis and fibrosis	1	7.14
Periductal mastitis	1	7.14
Total	14	100.0

Intraductal carcinoma (IDC) represented more than 80% of malignant cases. Histopathological grade was available in 67 cases. No grade 1 cases were encountered in the study. Lymph node (LN) status was available for 70 cases; 35 (50%) had ≤3 and the other 50% had >3 L.N involved. Data on tumor size were available for 55 patients; 35 (63.6%) had a tumor ≤ 5cm and 20 (36.4%) had a tumor >5cm. There was no correlation between histopathological grade and tumor size ($p = 0.4$) or number of LN involved ($p = 0.9$). Also, there was no correlation between tumor size and number of LN involved ($p=0.7$). Cell cycle kinetics and Cyclin B1 were simultaneously analyzed in 34 breast cancer tumors. Cyclin B1 was expressed in only5,50 cases (10%) by WB technique whereas FC could detect positive Cyclin B1 in 17 out of the 34 cases (50%). Fourteen cases were diploid and 20 were aneuploid. Thirteen cases (38.2%) showed an S phase of ≥10%. Table 2 shows the relationship between cyclin B1, S-phase and ploidy status. Cyclin B1 expression tended to be associated with S phase fraction ≥ 10% although insignificant ($p=0.5$), and with aneuploid rather than diploid tumors ($p=0.2$). However, Cyclin B1 expression was significantly associated with tumors with high G2M phases ($p=0.05$, table 3, Figure 2).

Table 2: Relation of S-phase fraction and Ploidy status to cyclin B1 MFCR

	Mean Cyclin B1 (±SD)	P value
S < 10%	2.31 (1.59)	0.5
S≥ 10%	2.61 (2.22)	
Diploid	1.93 (1.24)	0.2
Aneuploid	2.27 (2.09)	

Table 3: Association between G2M status and cyclin B1

G2M Phase	Cyclin B1	
	positive	negative
High (n=18)	13 (72.2%)	5 (27.8%)
Normal (n=16)	4 (25%)	12 (75%)

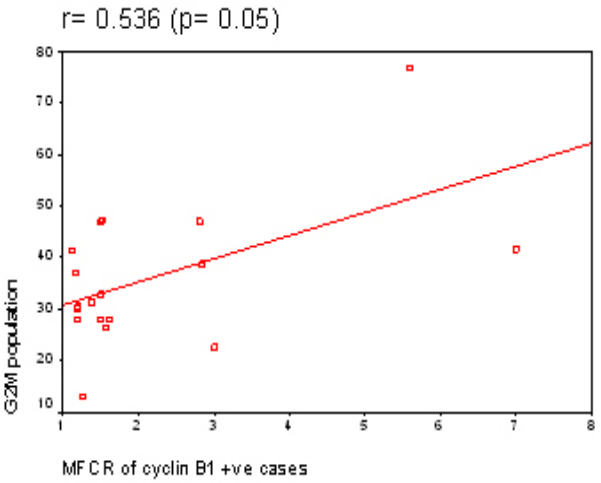


Fig. 2: Correlation between MFCR of + ve cyclin B1 and G2M population % in breast cancer cases.

Cyclin B1 was not correlated to age groups, tumor histopathological type, grade, size, number of LN involved, or hormone receptor status. Tumors with high G2M phases showed higher cyclin expression. DNA

ploidy status was not correlated to age, histopathological type, tumor size or grade, number of LN involved or hormone receptor status. However, S-phase fraction was significantly associated with -ve ER (p= 0.033) and PR (p= 0.043) as shown in table 4.

Table 4: Relation between S-phase fraction and the clinocopathological factors (ER and PR) in the breast cancer cases.

	S-phase fraction<10	S-phase fraction≥10	P value
	NO. (%)	NO. (%)	
Estrogen Receptor			
-ve(13)	6 (46.2)	7 (53.8)	0.033*
+ve(11)	10 (90.9)	1 (9.1)	
Progesterone Receptor			
-ve	5 (45.5)	6 (54.5)	0.043*
+ve	11 (84.6)	2 (15.4)	

Tissue content of putrescine, spermidine and spermine were evaluated in 45 malignant cases, 13 with benign lesions as well as in 16 control tissue specimens. The three studied polyamines and the total polyamine levels were significantly elevated in the malignant group cases compared to benign cases or control group (p< 0.001), (Table 5). Correlation studies between biochemical

Table 5: Levels of Polyamines (in nmol/100 mg tissue) in the three studied groups.

	Malignant N =45	Benign N = 13	Control N =16	P-value
Putrescine				
Mean± SD	4.7±5.8	0.5±0.3a	0.4±0.2b	< 0.001*
Spermidine				
Mean± SD	30.5±19.3	2.5±2.0a	3.6±2.3b	< 0.001*
Spermine				
Mean± SD	54.8±30.8	2.8±1.9a	2.4±1.3b	< 0.001*
Total polyamines				
Mean± SD	90.0±46.9	5.8±2.8a	6.4±3.1b	< 0.001*

*Significant

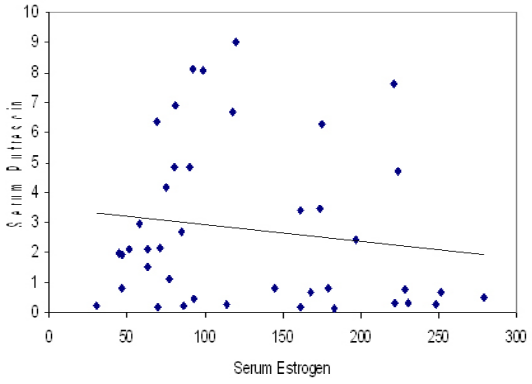


Fig. 3: Correlation between serum putrescine and serum estrogen in breast cancer cases (r = - 0.424, p = 0.03)

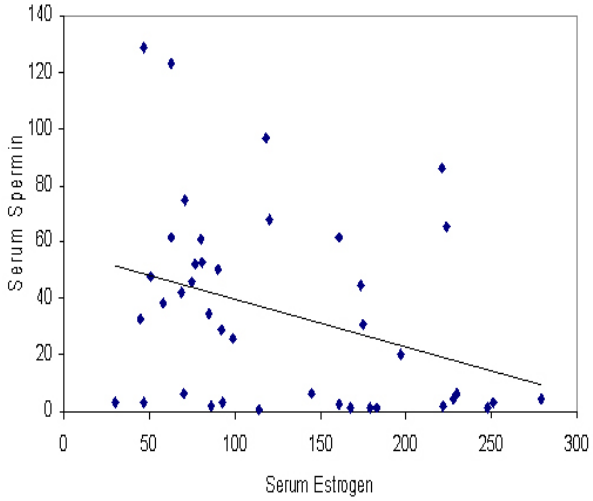


Fig. 4: Correlation between serum spermidine and serum estrogen in breast cancer cases (r = -0.450, p =0.021)

parameters showed a weak negative significant correlation between estrogen and both putrescine (r = -0.424, p =0.031, Figure 3) and spermidine (r = -0.450,0.021, Figure 4). Multivariant analysis was done to find out the clinical value of the studied biochemical parameters and cyclin B1 relative to the traditional prognostic factors. ROC curve was used to determine the cut off for each parameter that give highest sensitivity, specificity and total accuracy (Table 6). Polyamines showed the best predictive value with >92% accuracy whereas IGF1,

Table 6: Measurement of accuracy of the studied markers in breast cancer groups.

	Cut off	Sensitiviy %	Specificity %	PPV %	NPV %	Accuracy %
Growth factor	147.84	53.8	41.7	44.0	41.4	42.6
Estrogen	84	65.2	23.1	42.9	40.0	42.0
Progestron	0.35	73.9	23.1	45.9	50.0	46.9
Puterscine	0.81	95.1	93.3	93.0	92.3	92.9
Spermidine	7.5	95.1	93.3	97.5	87.5	94.6
Spermine	6.2	95.1	93.3	97.5	87.5	94.6
Total Polyamines	11.9	95.1	93.3	97.5	87.5	94.6

Table 7: Relation between serum estrogen level and the clinicopathological factors in the breast cancer cases.

Estradiol(ngimi)	Median	Range	p-value
Age groups			
≤45	105.0	5.0-221.0	0.016
> 45	72.5	26.0-224.0	
Estrogen receptor			
-ve	71.0	5.0-175.0	0.05
+ve	111.0	45.0-224.0	
Progesterone receptor			
-ve	71.0	5.0-175.0	0.016
+ve	118.0	45.0-224.0	
Histological type			
IDC	81.0	5.0-224.0	0.45
ILC	63.0	37.0-174.0	

estrogen and progesterone showed values <50%. High Serum estrogen was significantly associated with ER/ PR +ve tumors and age group <45 yrs (Table 7). Intralobular carcinoma cases were associated with higher total polyamine levels than intraductal carcinoma cases and the difference was statistically significant (p= 0.05). Spermidine levels were significantly higher in cases with tumors larger than 5cm in diameter (p = 0.028). On the other hand, spermidine levels were lower in ER +ve tumors compared to ER -ve ones (p = 0.049). Median spermidine levels in intralobular carcinoma cases were more than double that of intraductal carcinoma cases, but

Table 8: Relation between tissue spermidine level (nmol/100 mg tissue) and the clinicopathological factors in the breast cancer cases.

Spenmidine	Median	Range	p-value
Estrogen receptor			
-ve	30.1	5.4-77.6	0.049*
+ve	15.2	4.9-61.0	
Tumor size			
≤5 cm	17.7	4.9-77.6	0.028*
> 5 cm	33.5	15.2-65.7	
Histopathologic type			
IDC	24.1	4.9-76.4	0.115
ILC	52.0	15.2-77.6	

*Significant

the difference was not statistically significant (Table 8). Spermine levels were not correlated to any of the tissue parameters studied. By FCM, cyclin B1 was detected in 17 out of 34 cases (50%) however; it was detected in only 5 malignant samples among 50 cases by WB (10%). On the other hand, Ehrlich-Ascites carcinoma and HebG2 cell line showed over expression of cyclin B1 when subjected to WB. Their intense bands corresponded to the size of cyclin B1 (62KD, Figure 5) in both cell lines and breast cancer tissues. By FCM, cyclin B1 was found +ve in both types of cell lines (Figure 6). Serum levels of IGF1, tumor markers, serum estrogen and progesterone are shown for both age categories in tables 9 and 10. Figure 7 shows the distribution of IGF1 among different groups studied. Serum CA15.3 level was significantly correlated with cases having tumor size with ≥ 5cm (r= 0.349, p= 0.02, Figure 8). Serum levels of progesterone were correlated positively to serum estrogen levels (r = 0.326, p = 0.022, Figure 9)

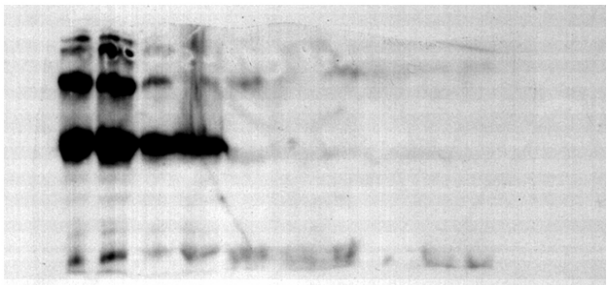
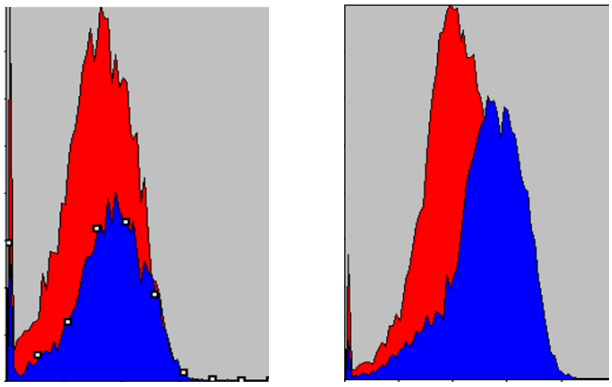


Fig. 5: Western blotting detection of cyclin B1 in cell lines lysate. Lanes 1 and 3 (Ehrlich), Lanes 2 and 4 (HebG2), Lanes 5-10: negative cyclin B1 in malignant breast samples.



a b

Fig. 6: flow cytometry Histograms of cyclin B1
(a) Breast cancer sample with 10% gated cyclin B1 positive with MFCR of 5.7.
(b) HebG2 cell line with 67% gated cyclin B1 positive cells with MFCR of 4.7.

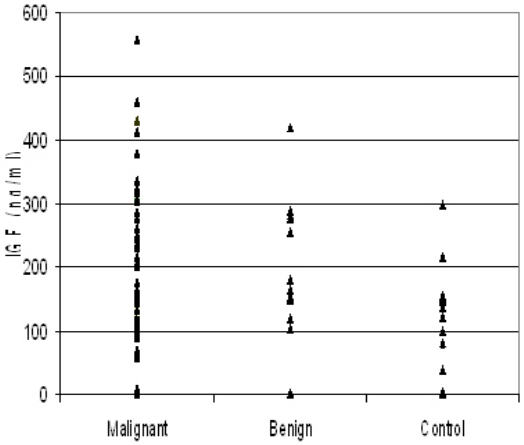


Fig. 7: Distribution of individual values in control, benign and Malignant groups for IGF1.

Table 9: Biochemical characteristics of the three studied groups (age group ≤45 y).

	Malignant N = 20	Benign N = 1	Control N = 14	p-value
Growth factor (ng/ml)				
Mean± SD	194.08±131.3	208.1±96.0	153.0±72.2	0.532
Serum CEA (ng/ml)				
Mean± SD	4.39±8.87	1.1±0.7	1.9±1.7	0.038*
Serum CA 15.3 (U/ml)				
Mean± SD	22.43±24.69	11.7±5.2	19.3±7.9	0.061
Serum estrogen (ng/ml)				
Mean± SD	118.25±58.81	168.0±63.7	123.4±78.4	0.082
Serum progesterone (ng/ml)				
Mean± SD	1.29±1.69	9.8±14.0	3.0±4.4	0.027*

* Significant

Table 10: Biochemical characteristics of the three studied groups (age group > 45 y)

	Malignant N =40	Control N = 5	p-value
Growth factor (ng/ml)			
Mean± SD	187.7±105.6	109.4±40.5	0.212
Serum CEA (ng/ml)			0.863
Mean± SD	3.1±3.4	3.7±4.5	
Serum CA 15.3 (U/ml)			0.228
Mean± SD	25.6±34.7	9.7±1.9	
Serum estrogen (ng/ml)			0.061
Mean± SD	81.3±42.4	38.5±12.0	
Serum Progesterone (ng/ml)			0.259
Mean± SD	0.5±0.7	0.5±0.1	

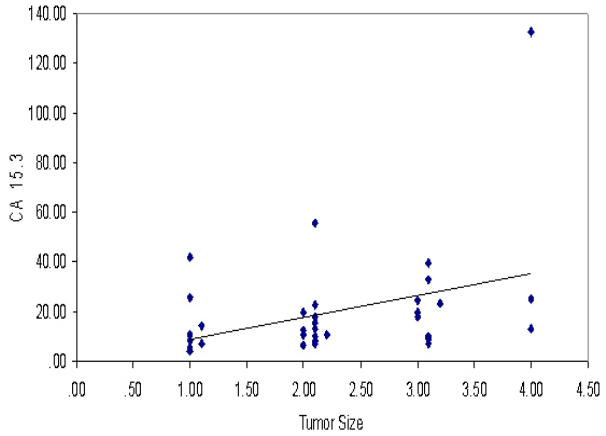


Fig. 8: Correlation between serum CA 15.3 and tumor size in Breast cancer cases (r = 0.349, p = 0.025)

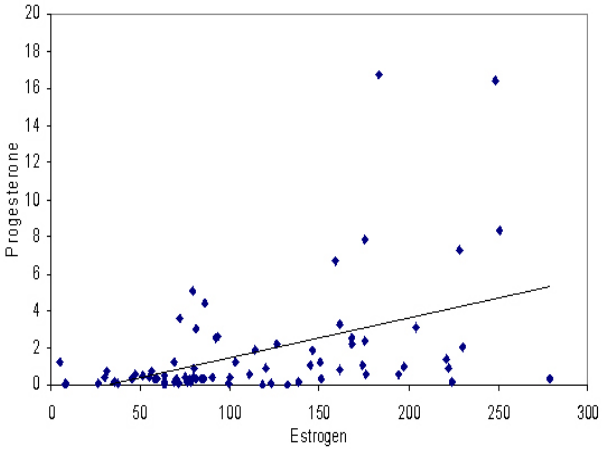


Fig. 9: Correlation between serum progesterone and serum estrogen in Breast cancer cases (r = 0.326, p = 0.022)

DISCUSSION

In this work, a comprehensive study of various prognostic markers was performed on cancer patients compared to cases with benign breast lesions and normal controls.

In the present study, breast cancer tissues were found to contain significantly higher concentrations of polyamines than benign and normal tissue (p=0.001) showing a 3-4 fold increase of all three free polyamines. This finding is in agreement with other studies reporting correlations between polyamine content of breast tissue and their causal link with rapid rates of proliferation of breast cancer[21]. A significant association was encountered between total polyamines and histological type being higher in ILC compared to IDC (p=0.05). Significant correlation was also encountered between spermidine and tumor size, being higher in tumors> 5cm (p= 0.04) and between spermidine and ER, being higher in ER –ve tumors. On the other hand, a significant negative correlation was encountered between these tissue polyamines and estrogens. Several studies on breast cancer cells suggested an interrelation between estrogens and polyamines[22] and suggested a specific role of polyamines in the interaction of ER with other activator proteins [11]. It was also postulated that increased polyamine levels were associated with aggressive forms of breast tumors.[23]

Polyamines showed high sensitivities, specificities and accuracy (Table 6). However, as tumor markers, in spite of the high sensitivity and specificity, they were claimed to be neither specific nor reliable for the early detection of cancer[24]. Still many aspects of their functions remain elusive and difficulties inherent to their analysis, by sophisticated HPLC methods, and the lack of standardization, have hampered the transit from the research to the standard clinical laboratory domain[25]. Polyamines could however be good markers of breast cancer proliferative activity (sensitivities: 95.1% and specifities: 93.3%) correlated to ER, tumor size and tumor grade.

Fifty percent (50%) of studied cases showed positivity of cyclin B1 by FCM while only 10% were positive by WB. All positive cases by WB were also found positive by FCM. Although WB is of high sensitivity as the chemiluminescence detection system used enhances this sensitivity along with the high specificity of purified MoAb against cyclin B1; the possibility of obtaining G2M cells from breast cancer tissues for WB is very small which clarify the discrepancy between both techniques and makes FCM superior in detecting low abundant proteins like cyclin B1[26]. Furthermore, cytoplasmic localization during G2 and nuclear translocation at the beginning of M phase are involved in the biological activities of cyclin B1[27]. In this respect, cyclin B1 immunoreactivity, localized both at cytoplasmic and nuclear levels, could affect its evaluation. Using FCM, cyclin B1 and cell cycle analysis were performed on 34 cancer breast cases and revealed that 71.4 % of grade 3 tumors and only 32% of grade 2 tumors showed ≥ 10% S-phase fraction. A high percent S-phase is known to be a major predictor of poor prognosis[28]. In this work, cyclin B1

positivity was found to be correlated with tumor grade showing 40 % positivity in grade 2 tumors and 71.4% in grade 3 reflecting its relation to the clinicopathological aggressiveness of the tumor.

As a mitotic cyclin, cyclin B1 appeared to be highly associated with tumors of high G2M and was positive in 13/18 (72.2%) cases with high G2M fraction. However, 5 cases with high G2M didn't express cyclin B1. Our explanation is that one main value of studying cyclin B1 in breast cancer is to discriminate between tumors with high G2M phase from a tetraploid population which is important to verify if one or the other is of prognostic relevance. The inverse relation between cyclin B1 and tetraploid population may also reflect the clinicopathological aggressiveness of the tumor. As Cyclin B1 rapidly disappears from the nuclei during late mitosis[29], FCM analysis of cyclin B1 can demonstrate the occurrence of cell cycle dependent translocation[27] and disappearance of the protein in every tested tissue tumor with the frequency depending on the variability of proliferative activity in cell yields in light of intertumoral heterogeneity[5]. FCM analysis detects individual viable cells where measurements are made separately on each cell within the suspension, while in WB, the number of cyclin-positive cells, if any, did not give enough proteins to be detected. Furthermore, the rapid degradability of the cyclin B1 at the end of mitosis, which itself occupies a small fraction of cell cycle time[30], makes it a difficult task to target substantial amounts of this protein.

The two types of cultured cells studied in this work showed an overexpression of cyclin B1 by WB and FCM. HebG2 cell lines gave 67% cyclin B1 positive (MFCR: 4.7) while Ehrlich Ascites-cultured cells gave only 27% (MFCR: 5.7). The G2M fraction of both cultured cells exceeded the 90%. FCM provided reliable quantitation of relative levels of cyclin B1 in intact cells while remaining sensitive to total amount of specific protein as in WB. It additionally allows the direct determination of both the frequency of cell expressing cyclin B1 as well as reliable to its intensity in cell populations without synchronization. This implies the superiority of FCM over WB in studying cyclins.

Aneuploidy was encountered in 57.6% of cases, SPF≥10% was encountered in 39.4% and high G2M was encountered in 18 out of 34 cases (52.9%). Both ER-ve and PR-ve status were significantly associated with the high SPF (≥10%) ($p = 0.03$ and 0.04 respectively). Aneuploidy and high percentage of S phases are useful in predicting prognostic parameters in stage I breast cancer patients. Abnormal ploidy, High SPF, and Cyclin B1 positivity predict a worsened disease-free or overall survival in most adult malignancies.[31]

With regards to the investigated prognostic factors, mean cyclin B1 positive cells (shown by MFCR) was higher in aneuploid tumor than in diploid tumors (MFCR: 2.27 & 1.93, respectively). The mean cyclin B1 positive cells was not significantly higher in S-phase ≥ 10% than in S-phase <10% (MFCR: 2.61 and 2.31, respectively). This is in agreement with others who concluded that abnormal cyclin expression in aneuploid conditions could reflect an increased cell growth fraction, as a consequence of an enhanced capacity of the tumor cells to respond to growth factors.[18]

In our study, IGFl was insignificantly increased in breast cancer patient when compared to age-matched control group. Epidemiological studies indicated an association between elevated serum levels of IGF1 and an increased risk for breast cancer[8]. Descriptive studies of fibrocystic diseases which may harbor hyperplastic lesions have reported expression of IGF1 in 50% of cases[32]. It was speculated that IGFs play a role in the development and progression of cancer as it could act as mitogen promoting the growth of breast tumors cells. It was also shown that an increased delivery of IGF1 to tissues may be detrimental by stimulating tumor growth in breast cancer patients[33]. It was suggested that IGF1 bioavailability mediated by its binding proteins may participate in both breast carcinogenesis and selection of more aggressive breast carcinoma clones[32]. However, this effect was found among premenopausal women in particular. In our study, one case with recurrent IDC showed the highest serum IGF1 level (557ng/ml), a case that deserves meticulous follow up. Recently, a fully human monoclonal antibody to IGF1 receptor was shown to block ligand-dependent signaling and inhibits human tumor growth in vivo[34] which makes IGF1 a candidate for targeted therapy[8]. Plasma IGF1 concentrations may be useful in the identification of women at high risk of breast cancer and in the development of risk reduction strategies.

In this study, serum estradiol level showed no significant change when compared to control group ($p< 0.06$). Lack of statistical significance could be attributed to the small number of control group. A positive association was found between high serum estradiol levels and breast cancer risk. Estrogen levels vary greatly depending on genetic susceptibility factors, The prevalence of 16 α-hydroxylation of estradiol, a variant of one gene called cyp17 gene is an example of polymorphism strongly associated with the risk of breast cancer.[35]

A positive relation was encountered between high estrogen level and young age (≤ 45 years). Estrogen level was also found to be correlated with ER +ve ($p = 0.05$) and PR +ve ($p = 0.016$), and was significantly higher when both receptors were positive ($p = 0.009$). This suggests that estrogens have a role in the stimulation of both ER and PR in breast adenocarcinoma[36]. As regards serum progesterone level, there is a significant decrease in <45 years breast cancer patients when compared to the

age matched benign group (p =0.02). This is in agreement with data suggesting that a relatively low progesterone secretion might favor the development of cancer. It was also observed that a low progesterone/estrogen ratio and anovulatory cycles are more frequent in breast cancer patients than in normal population. On the other hand, there was a significant correlation between serum progesterone level and ER and PR which is in accordance with previous reports.[37]

Evaluation of the different measurements of accuracy of the studied markers was done using the cut-off values based on ROC curve for IGF1, CEA, CA 15.3, estrogen and progesterone. Measured accuracies were as follows: IGF1: 42%, CEA: 57%, CA15.3: 52%, estrogen: 42%, progesterone: 47%, putrescine: 92%, spermidine: 95%, spermine: 95%, and polyamines: 95% (data showed in table 6. Previous obtained reports[38] for markers sensitivies as20. 5% for CA 15-3 and 10.7% for CEA concluded the need of tumor markers of greater clinical utilities as serum IGF 1 or tissue polyamines.

CONCLUSION

- The plausible relationships between hormones, growth factors, cyclins and polyamines in human breast cancer seems to be more complex than their counterpart previously observed in cultured cells. An array of factors intervenes in defining the behavior, kinetic and outcome of breast cancer and the heterogeneity of breast carcinoma may worsen this complexity.

- Cyclin B1 is associated with high proliferative and more aggressive tumors. This needs further studies on larger scale of cases using multiparametric FCM to obtain multiple simultaneous data on different cell populations. Cyclin B1 assessment by FCM proved superior to Western Blotting in this context.

REFERENCES

1. Piccart MJ, Awada A. State-of-the-art chemotherapy for advanced breast cancer. Semin Oncol 2000 Oct;27(5 Suppl 9):3-12.

2. Eifel P, Axelson JA, Costa J, Crowley J, Curran WJ,Jr, Deshler A, et al. National Institutes of Health Consensus Development Conference Statement: Adjuvant therapy for breast cancer, November 1-3, 2000. J Natl Cancer Inst 2001 Jul 4;93(13):979-89.

3. Kornek GV, Haider K, Kwasny W, Raderer M, Schull B, Payrits T, et al. Treatment of advanced breast cancer with docetaxel and gemcitabine with and without human granulocyte colony-stimulating factor. Clin Cancer Res 2002 May;8(5):1051-6.

4. Sherr CJ. Mammalian G1 cyclins. Cell 1993 Jun 18;73(6):1059-65.

5. Megha T, Lazzi S, Ferrari F, Vatti R, Howard CM, Cevenini G, et al. Expression of the G2-M checkpoint regulators cyclin B1 and P34CDC2 in breast cancer: A correlation with cellular kinetics. Anticancer Res 1999 Jan-Feb;19(1A):163-9.

6. Thomas T, Thomas TJ. Regulation of cyclin B1 by estradiol and polyamines in MCF-7 breast cancer cells. Cancer Res 1994 Feb 15;54(4):1077-84.

7. Dickson RB, Lippman ME. Growth factors in breast cancer. Endocr Rev 1995 Oct;16(5):559-89.

8. Furstenberger G, Morant R, Senn HJ. Insulin-like growth factors and breast cancer. Onkologie 2003 Jun;26(3):290-4.

9. Thomas T, Thomas TJ. Polyamines in cell growth and cell death: Molecular mechanisms and therapeutic applications. Cell Mol Life Sci 2001 Feb;58(2):244-58.

10. Thomas T, Shah N, Klinge CM, Faaland CA, Adihkarakunnathu S, Gallo MA, et al. Polyamine biosynthesis inhibitors alter protein-protein interactions involving estrogen receptor in MCF-7 breast cancer cells. J Mol Endocrinol 1999 Apr;22(2):131-9.

11. D'Agostino L, Di Luccia A. Polyamines interact with DNA as molecular aggregates. Eur J Biochem 2002 Sep;269(17):4317-25.

12. Love RR, Astrow SH, Cheeks AM, Havighurst TC. Ornithine decarboxylase (ODC) as a prognostic factor in operable breast cancer. Breast Cancer Res Treat 2003 Jun;79(3):329-34.

13. Huber M, Poulin R. Permissive role of polyamines in the cooperative action of estrogens and insulin or insulin-like growth factor I on human breast cancer cell growth. J Clin Endocrinol Metab 1996 Jan;81(1):113-23.

14. Bidwell GL,3rd, Raucher D. Enhancing the antiproliferative effect of topoisomerase II inhibitors using a polypeptide inhibitor of c-Myc. Biochem Pharmacol 2006 Jan 12;71(3):248-56.

15. Sweeney K, Musgrove A, Watts C, Sutherland R. Cyclins and breast cancer. In: Dickson RB, Lippman ME, editors. Mammary tumor cell cycle, differentiation and metastasis: Advances in cellular and molecular biology of breast cancer (Cancer treatment and research): Kluwer Academic Publishers; 1996. p. 141.

16. Verkoelen CF, Romijn JC, Schroeder FH, van Schalkwijk WP, Splinter TA. Quantitation of polyamines in cultured cells and tissue homogenates by reversed-phase high-performance liquid chromatography of their benzoyl derivatives. J Chromatogr 1988 Apr 8;426(1):41-54.

17. Darzynkiewicz Z, Gong J, Traganos F. Analysis of DNA content and cyclin protein expression in studies of DNA ploidy, growth fraction, lymphocyte stimulation, and the cell cycle. Methods Cell Biol 1994;41:421-35.

18. Collecchi P, Santoni T, Gnesi E, Giuseppe Naccarato A, Passoni A, Rocchetta M, et al. Cyclins of phases G1, S and G2/M are overexpressed in aneuploid mammary carcinomas. Cytometry 2000 Aug 15;42(4):254-60.

19. Bronstein I, Voyta JC, Murphy OJ, Bresnick L, Kricka LJ. Improved chemiluminescent western blotting procedure. BioTechniques 1992 May;12(5):748-53.

20. Dawson B, Trapp RG. Basic and clinical biostatistics. 3rd ed. Boston: Lange Medical Books/McGraw-Hill; 2001.

21. Canizares F, Salinas J, de las Heras M, Diaz J, Tovar I, Martinez P, et al. Prognostic value of ornithine decarboxylase and polyamines in human breast cancer: Correlation with clinicopathologic parameters. Clin Cancer Res 1999 Aug;5(8):2035-41.

22. Marton LJ, Pegg AE. Polyamines as targets for therapeutic intervention. Annu Rev Pharmacol Toxicol 1995;35:55-91.

23. Leveque J, Foucher F, Bansard JY, Havouis R, Grall JY, Moulinoux JP. Polyamine profiles in tumor, normal tissue of the homologous breast, blood, and urine of breast cancer sufferers. Breast Cancer Res Treat 2000 Mar;60(2):99-105.

24. Lawton F, Griffin M, Slack J, Blackledge G. Urinary polyamine excretion patterns in patients with epithelial ovarian cancer. Gynecol Obstet Invest 1989;28(4):212-4.

25. Gugliucci A. Polyamines as clinical laboratory tools. Clin Chim Acta 2004 Jun;344(1-2):23-35.

26. Darzynkiewicz Z, Gong J, Juan G, Ardelt B, Traganos F. Cytometry of cyclin proteins. Cytometry 1996 Sep 1;25(1):1-13.

27. Li J, Meyer AN, Donoghue DJ. Nuclear localization of cyclin B1 mediates its biological activity and is regulated by phosphorylation. Proc Natl Acad Sci U S A 1997 Jan 21;94(2):502-7.

28. Kallioniemi OP. Comparison of fresh and paraffin-embedded tissue as starting material for DNA flow cytometry and evaluation of intratumor heterogeneity. Cytometry 1988 Mar;9(2):164-9.

29. Kawamoto H, Koizumi H, Uchikoshi T. Expression of the G2-M checkpoint regulators cyclin B1 and cdc2 in nonmalignant and malignant human breast lesions: Immunocytochemical and quantitative image analyses. Am J Pathol 1997 Jan;150(1):15-23.

30. Widrow RJ, Rabinovitch PS, Cho K, Laird CD. Separation of cells at different times within G2 and mitosis by cyclin B1 flow cytometry. Cytometry 1997 Mar 1;27(3):250-4.

31. Aziz DC, Peter JB. DNA ploidy and cell-cycle analysis: tools for assessment of cancer prognosis. J Clin Lab Anal 1991;5(6):422-38.

32. Nguyen Q, Jameson LJ. Cell cycle. In: Rosenzweig A, Jameson LJ, editors. Principles of molecular medicine. 1st ed. Totowa, New Jersey: Humana Press; 1998. p. 66-70.

33. Helle SI, Lonning PE. Insulin-like growth factors in breast cancer. Acta Oncol 1996;35 Suppl 5:19-22.

34. Burtrum D, Zhu Z, Lu D, Anderson DM, Prewett M, Pereira DS, et al. A fully human monoclonal antibody to the insulin-like growth factor I receptor blocks ligand-dependent signaling and inhibits human tumor growth in vivo. Cancer Res 2003 Dec 15;63(24):8912-21.

35. Haiman CA, Hankinson SE, Colditz GA, Hunter DJ, De Vivo I. A polymorphism in CYP17 and endometrial cancer risk. Cancer Res 2001 May 15;61(10):3955-60.

36. Fishman J. Endocrine participation in the etiology of breast cancer. In: Harris JR, Hellman S, Henderson IC, Kinne DW, editors. Breast diseases. Philadelphia: JB Lippincott company; 1987. p. 103.

37. Lumachi F, Basso SM, Brandes AA, Pagano D, Ermani M. Relationship between tumor markers CEA and CA 15-3, TNM staging, estrogen receptor rate and MIB-1 index in patients with pT1-2 breast cancer. Anticancer Res 2004 Sep-Oct;24(5B):3221-4.

38. Heinze T, Schurenkamper P, Minguillon C, Lichtenegger W. Mammary serum antigen (MSA), Ca 549, CA 15-3 and CEA in breast cancer preoperative sensitivity and correlation to prognostic factors. Anticancer Res 1997 Jul-Aug;17(4B):2953-4.