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PREDICTIVE STUDY ON FEMALE BREAST CANCER USING SOME BIOLOGICAL MARKERS

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ABSTRACT

Breast cancer is the most common cancer worldwide. The size, stage, rates of growth and other factors control the type of treatment. The use of markers for cell proliferation and the new techniques such as flowcytometry for analysis of DNA pattern have been among the most frequently used techniques for the detection and the prediction of cancer outcome. The proliferation biomarker Ki-76 antigen and cell cycle progression are prognostic factors for breast cancer. The aim of this study was to evaluate the effectiveness and prognostic value of previous markers on mononuclear leukocytes obtained from peripheral blood and malignant tissue of breast cancer patients to identify the high-risk patients who may benefit from the adjuvant systemic therapy. These results revealed that the combination of high expression Ki67, SPF, G2/M, high grade and Aneuploidy with low values of Go/1 resulted in poor prognosis of patient of breast cancer. This combinatorial approach may also prove a valuable prognosis and assess response to anticancer treatment and the subsequent clinical outcomes.

Key words: Breast cancer**,** Ki-67,Cell cycle**,** Prognostic factor and flowcytometry

INTRODUCTION

Breast cancer is one of the most common human malignancies, which accounts for 23% of the total new cancer cases and 14% of the total cancer deaths and the second leading cause of cancer deaths in women today **(Rennert, 2006 and Jemal, et al., 2011).** Human breast cancer defined as heterogeneous disease according to histopathologically, molecularly and phenotypically basics. The main challenge today is to find factors which could predict the patients who have a tumor with aggressive nature. There are standard prognostic factors of breast cancer such as axillary lymph node (LN) status, tumor size, histological grade, clinical stage, estrogen receptor (ER) positivity, progesterone receptor (PR) positivity, and HER2/neu Oncogene status(Human epidermal growth factor receptor). Breast cancer treatment could be based on these factors which have been used to identify the high-risk patients who may benefit from the adjuvant systemic therapy. Lymphocytes-infiltrating (LI) (monocytes, B and T lymphocytes) may represent an essential pathophysiological factor in the development, progression and prognosis of breast cancer **(Coussens, et al., 2002 and Meylan, et al., 2006).** Bad prognosis of patients with advanced stage breast cancer is mainly due to the typical fast metastasis that is rarely detected at the early stages **(Fisher, et al., 1991 and Bardou, et al., 2003)**.

Predicting the clinical course of breast tumours is very problematic because is an unpredictable disease with a heterogeneous natural history. Some patients are cured by local therapy and survive for many years; others suffer from rapid deterioration with early recurrence of their disease followed shortly by death. Predicting with different prognostic factors would be obviously useful to identify individual patients who are at high or low risk for relapse in order to plan the appropriate management of the patient's breast cancer **(Spyratos, 1993 and Masood, 1997).**

Cytometric quantitation of nuclear DNA content can assist in the diagnosis and grading of malignant tumors. Nuclear DNA content strongly correlated to histopathologic grade of ductal carcinoma and has a significant value in prognosis of breast cancer **(Erhardt & Auer, 1986 and Bocking, et al., 1995).**

(Wolman, et al., 1994 and Ross, 1996). illustrated that in normal tissues and most low-grade or slowly proliferating neoplasm, approximately 85% of the cell population forms the G0/G1 peak and

15% of the cells are in the S and G2M phases with DNA index(1.0). Ploidy status of tumors is based on the DNA index (D1)which refer to the ratio of abnormal to normal DNA **(Spyratos, 1993)**. Abnormal cell proliferation which results from deregulation of the cell cycle is fundamental in tumorigenesis. In abnormal cell proliferation, no diploid cell populations (tumor), featuring a DNA index of the G0/G1 main peak might be less than 1.0 or greater than 1.0 and significantly increased G2M phases and S-phase resulted in a deceptively high rate of proliferation in breast carcinoma. S-Phase fraction (SPF) reflects proliferative activity and estimates the neoplastic growth rate and had prognostic value in breast cancer. Low SPF(S-phase fraction) is associated with an excellent prognosis in breast patients **(Michels, et al., 2000 and Evan, et al., 2001)**. According to **(Wong, et al., 1999; Bracko et al., 2001 and Tsutsui, et al., 2001)** the tumors with DNA peaks within diploid limits have a more favorable prognosis than those with aneuploid peaks. High grade breast cancer were more likely to be aneuploid than others. Aneuploid tumors were associated with a poorer prognosis than diploid tumors in breast cancer patients.

Proliferation rate is a feature of tumors that has been illustrate the potential of malignancy potential and is an important prognostic factor which forms part of the grading classification and is associated with many other prognostic biomarkers. The Ki-67 monoclonal antibody has been developed to recognize the Ki-67 nuclear antigen as immunogen that is present in proliferating cells (but absent in resting cells) used in evaluating cellular proliferation rates of malignant tumor **(Gerdes, et al., 1983 and Cooper, et al., 1998)**. Ki-67 antigen is a proliferation antigen and one of several cell-cycle regulating proteins. Ki-67 is expressed during all phases of the cell cycle except G0 (resting phase).**(Scagliotti, et al., 1993**; **Bridger, et al., 1998 and MacCallum, et al., 2000)** showed that cellular expression of Ki-67 used as marker of tumor proliferation and had a role in organization , maintenance of the architecture of DNA and synthesis of ribosomes during mitosis. Because most of the Ki-67 positive cells are in the cell cycle, Ki-67 labelling index, fraction of Ki-67 positive nuclei of all nuclei reflects the fraction of proliferating cells. The prognostic value of Ki-67 has been reported in a wide variety of benign and malignant tumours including lung carcinoma, prostatic carcinoma, colorectal carcinoma**,** hepatocellular carcinoma **(Gatter, et al., 1986; Raymond, et al., 1988; Shepherd, et al., 1988 and Grigoni, et al., 1989)**, respectively. Many studies

demonstrated the association between a high Ki-67 labelling index, histological grade and large tumor size in breast carcinoma; also it has prognostic value in breast cancer patients **(Isola, et al., 1990 and Pietilainen, et al., 1996).**

There is growing evidence that interaction of stromal and immune cells with normal or malignant epithelial cells is pivotal for the development and progression of cancer. Several reports indicate that tumour-infiltrating leucocytes may represent an essential pathophysiological factor in the development and progression of breast cancer **(Coussens, et al., 2002 and Meylan, et al., 2006).** Lymphocyte infiltration (LI) is an essential pathophysiological factor in the development and progression of breast cancer. It was suggested as a marker of host antitumor immune response, but its importance in terms of pathophysiology and prognosis or treatment prediction remains controversial. In rapidly proliferating tumors LI has been shown to be a good prognostic indicator, correlating with lymph-node negativity, smaller tumor size, and lower grade **(Coronella-Wood, et al., 2003 and Kohrt, et al., 2005)**. Similarly, **(Ménard, et al., 1997)** have shown that lymphocyte infiltration of breast cancer had a strong positive prognostic value in patients**.**

The aim of the present work is to evaluate the effectiveness and prognostic value of some biomarker (cell cycle phases and Ki67 antigen) on mononuclear leukocytes obtained from peripheral blood and malignant tissue of breast cancer patients. Also to identify the high-risk patients who may benefit from the adjuvant systemic therapy. In addition, to predict tumor progression, recurrence and metastasis which might discovered after the standard surgical treatment.

PATIENTS AND METHODS

Tumor and patients data

In the present study, during the period between 2010 to 2012 year, seventy Egyptian female breast tumor patients of different etiology with ages ranged from 30 to 76 years with mean age of 54 years were used. Breast cancer patients group including 50 cases with median age of 54 years, ranged from 30 to 76 years. Breast benign tumor used as a control group includes 20 cases with median age of 41, ranged from 30 to 47 years. Patient`s clinicopathological data were retrieved from medical records. These data included sex, age, race, lifetime smoking history,

family history of breast cancer, date of diagnosis, date of surgery, adjuvant chemotherapy, pathological stage of tumor, histology and grade.

Sampling and tissue extracting

Tissue and tumour specimens (ductal carcinoma in situ, invasive ductal carcinoma) tumor tissues were obtained from surgical procedures then fixed in saline solution. Blood sample were collected on anticoagulant (EDTA) before and after chemohormonal therapy**.**

The DNA histogram and Ki-67 antigen immunoreactivity were measured by flowcytometry using a FACS calibar flowcytometer in (Becton Dickinson, sunnyvale, CA, USA)in Children Hospital , Mansoura University. The tissues were suspended in phosphate buffered saline containing EDTA and filtered suspension through a 25 µm nylon mesh then spin down at 1800 rpm to isolate the extracted tumor cells. The human peripheral leukocyte cells isolated from blood samples by using Lymphoflot. Both separated tumor cells and leukocyte fixed in ice-cold absolute ethanol overnight and divided into two parts for DNA cell cycle analysis and evaluation of Ki-67 immunoreactivity.

Detection and scoring methods

The first part of the suspension for DNA cell cycle analysis, cells were then washed with PBS (Phosphate buffer solution) for two times and stained with propidium iodide (PI) (50 µL) (Sigma Diagnostics) in the dark at room temperature for 30 min at a final concentration of 0.208 gm/mL according to the method of **(Vindelov, et al., 1983)**.

The second part was fixed with Dako Cytomation IntraStain Reagent A, K 2311 and incubated for 10 min., washed with PBS, then Permeabilization, with (DakoCytomation IntraStain Reagent B, K2311) and finally stained by using a method of slight modification with fluorescein isothiocyanate-conjugated Ki-67 monoclonal antibody, F 7268 (Dakopatts, Glostrup, Denmark), incubated in the dark at +4ºC for 15 min. and analyse on a flow cytometer **(Landberg, et a1., 1990)**. Flow cytometry has been demonstrated to be a useful method for detecting Ki-67 antigen with Monoclonal Anti-Human Ki-67 Antigen/FITC (MIB-1) for identification of cells expressing the Ki-67 antigen and assessing cellular proliferation in tumor cells **(Gerdes, et al., 1991 and Endl, et al., 2001).** The tumor proliferative activity, as evaluated by using the monoclonal antibody Ki-67, appears to be an effective indicator of the

prognosis for breast cancer **(Veronese, et al., 1993 and Kobayashi, et al., 1995).**

Data were analyzed using CELLFIT TM and LYSIS TM software (BectonDickinson and Company). The coefficient of variation (CV) of the G0/G1 peak .The aneuploid peak was judged at its S phase fraction; G2M apparently existed and could be analyzed by the software .The minimum value of the DNA index (ratio of the aneuploid G0/G1 peak to the reference diploid G0/G1 peak) was 1.10. The values of Ki-67 positivity of the G0/G1 peak of the diploid or aneuploid cell population were measured as a percent of its cell population over the upper limit that was obtained by the control monoclonal antibody (Dakopatts)

Statistical analysis:

Statistical analysis was performed using SPSS 17.0 software. All results calculated and expressed as mean ± SD.

RESULTS

Patient characteristics are summarized in Table 1.

Table (2) represents the measured parameters in different tested groups. In tumor, there were insignificant differences in DI (DNA index) and Diploidy% between breast cancer tissue samples and that found in control group ($P>0.05$) while significant differences in S-phase%, G0/1, G2/G1and,G2\M were observed. In addition there were a highly significant differences in Ki-67 and Aneuploidy in breast cancer tissue sample patient's compared with that of the control group (P=0.000). In addition there is a positive correlation between Ki-67 with both S-phase% and G2/M. In tested specimen before chemohormonal therapy(blood1) there were insignificant differences in S-phase% , DI , Diploidy% and G2/M while significant differences in G0/1 and G2/G1 were observed. where, there were a highly significant differences in Ki-67 and Aneuploidy between blood1 sample patient's compared with that of control group (P=0.000. In tested specimen before chemohormonal therapy (blood2). There were insignificant differences in G2/G1, DI and Diploidy %(P>0.05) and significant differences in G0/1 and G2/M (P=0.01) were observed. Where, there were a highly significant differences in Ki-67, S% and Aneuploidy between blood2 samples compared with that found in control group (P=0.000). On the other hand there is a positive correlation between Ki-67 with both S-phase% and G2/M.

Table (1): Demographic Parameters for Different Studied Groups (Patients and Controls Group).

Parameter	Value
Breast cancer patients group	
No. of cases	50
Age	
mean ± S.D	53.9±11.4
Range	30 – 76 years
Grade	
Gradell	40 patients (80%)
Grade lll	10 patients (20%)
Lymph node	
+ve Lymph node	36 (72%)
-ve lymph node	14 (28%)
ER (Estrogen receptor)	
+ve ER	30 (60%)
-ve ER	20 (40%)
PR (Progesterone receptor)	
+ve PR	36(72%)
-ve PR	14 (28%)
Menopausal	
Postmenopausal	36 (72%)
Premenopausal	14 (28%)
Tumor size	
T1	8 (16%)
T2	30(60%)
T3	12 (24%)
Benign tumor patients group	
No. of cases	20
Age	
mean±S.D	40.9±6.08
Range	30-47 years
Tumor size	Tx

T1: Tumor is in between 0.1cm to 2.0 cm in greatest dimension.
T2: Tumor is in between 2.0 cm to 5.0 cm in greatest dimension.
T3: Tumor is more than 5.0 cm in greatest dimension.
TX: Primary tumor cannot be assessed (benign tumor).

Table (2): Comparison between parameters in breast cancer tissue sample, blood1, blood2 and that from control (benign tumor).

parameter	Tumor (mean±S.D)			Blood 1•(before treatment) (mean±S.D)			Blood 2••(after treatment) (mean±S.D)		
Group	Patients (no. 50)	Benign (no. 20)	P	Patients (no. 50)	Benign (no. 20)	P	Patients (no. 48)	Benign (no. 20)	P
Ki 67	66.65 ± 16.65	23.45 ± 3.59	0.000**	47.93±20.80	26.28±4.66	0.000**	45.27±17.30	9.07±2.08	0.000**
Go /1	63.85±16.80	76.53± 2.30	0.01*	76.88±14.13	83.17±3.07	0.05*	91.09±6.06	96.28±2.91	0.01*
S %	24.84±15.28	17.70±1.54	0.05*	13.15±9.45	11.73±0.98	>0.05	4.79±3.14	2.00±1.79	0.001**
G 2/M	11.40±10.63	5.64± 1.70	0.01*	10.24±13.02	7.96±2.94	>0.05	4.52±4.13	1.39±0.86	0.01*
G2/G1	1.99±0.54	2.86±0.84	0.01*	1.89±0.45	2.60±0.78	0.05*	2.07±0.45	2.36±0.54	>0.05
DI	1.1±0.16	1.00±0.00	>0.05	1.06±0.29	1.00±0.00	>0.05	0.98±0.07	1.00±0.00	>0.05
Diploidy%	88.22±23.58	100.00±0.00	>0.05	94.95±15.34	100.0±0.00	>0.05	95.12±16.53	100.0±0.00	>0.05
Aneuploidy%	42.06±27.16	0.00±0.00	0.000**	42.12±22.03	0.00±0.00	0.000**	58.55±0.28	0.00±0.00	0.000**

Blood1• : The human peripheral leukocyte cells isolated from blood samples during surgery and before chemohormonal therapy.
Blood2•• : The human peripheral leukocyte cells isolated from blood sample after chemohormonal therapy .
*:significant. **:highly significant

Table (3): Comparison between parameters according to tumor size (T1, T2 and T3) in breast cancer tissue sample and benign tumor (Tx).

Parameter	Tumor size	No	mean±S.D.	P
Ki-67	T1	8	67.98±17.39	
	T2	30	64.75±18.09	0.000**
	T3	12	70.52±14.20	
	TX	20	23.45±3.95	
Go\1	T1	8	59.30±7.80	>0.05
	T2	30	64.91±18.43	
	T3	12	64.25±18.62	
	TX	20	76.52±2.3	
S%	T1	8	27.28±18.37	
	T2	30	22.41±14.84	>0.05
	T3	12	29.29±15.98	
	TX	20	17.69±1.5	
G2\M	T1	8	13.42±11.36	
	T2	30	12.68±12.17	
	T3	12	6.87±4.20	>0.05
	TX	20	5.64±1.79	
G2\G1	T1	8	2.05±0.66	
	T2	30	1.95±0.59	>0.05
	T3	12	2.06±0.37	
	TX	20	2.86±0.84	
DI	T1	8	1.18±0.25	>0.05
	T2	30	1.06±0.16	
	T3	12	1.01±0.04	
	TX	20	0.1±0.00	
Diploidy%	T1	8	94.18±8.33	>0.05
	T2	30	84.75±18.26	
	T3	12	92.94±7.05	
	TX	20	100.00±0.00	
Aneuploidy%	T1	8	11.64±8.54	>0.05
	T2	30	57.20±22.99	
	T3	12	42.31	
	TX	20	0.00±0.00	

T1: Tumor is in between 0.1cm to 2.0 cm in greatest dimension.
T2: Tumor is in between 2.0 cm to 5.0 cm in greatest dimension.
T3: Tumor is more than 5.0 cm in greatest dimension.
TX: Primary tumor cannot be assessed (benign tumor).
* significant. ** highly significant

Table (4): Comparison between parameters according to tumor grades in breast cancer tissue sample.

parameter	Tumor (mean±S.D)		
Group	**GII no. (%) 40(80%)**	**GIII no. (%) 10(20%)**	**P**
Ki-67	67.27±17.14	64.16±16.06	>0.05
Go /1	61.80±14.93	72.05±23.00	>0.05
S %	26.05±14.82	20.05±17.93	>0.05
G 2/M	12.28±11.19	7.89±8.00	>0.05
G2/G1	1.98±0.57	2.04±0.48	>0.05
Apoptosis	5.186±3.47	4.54±2.89	>0.05
DI	1.13±0.18	1.03±0.14	>0.05
Diploidy%	88.94±24.73	85.35±20.46	>0.05
Aneuploidy%	44.23±32.71	36.63±8.04	>0.05

GI: Well differentiated breast cell (there were not cases with GI).
GII: Moderately differentiated breast cell.
GIII: Poorly differentiated breast cell tend to grow and spread more aggressively.

Table (5): Comparison between parameters according to DNA ploidy in breast cancer tissue sample:

parameter	Group	No. of cases / (%)	Mean± SD	P
Ki-67	Diploid	(72%)	64.36±15.64	>0.05
	Aneuploid	(28%)	72.52±18.98	
Go\1	Diploid	(72%)	56.28±7.56	0.005**
	Aneuploid	(28%)	83.32±18.78	
S%	Diploid	(72%)	30.99±10.71	0.005**
	Aneuploid	(28%)	9.015±14.56	
G2\M	Diploid	(72%)	12.86±10.30	>0.05
	Aneuploid	(28%)	7.66±11.34	
G2\G1	Diploid	(72%)	1.99±0.64	>0.05
	Aneuploid	(28%)	2.00±0.00	
DI	Diploid	(72%)	1.00±0.00	0.01*
	Aneuploid	(28%)	1.25±0.22	
Diploidy %	Diploid	(72%)	100.00±0.00	0.005**
	Aneuploid	(28%)	57.94±27.16	

*:significant. **:highly significant

Table (3) represents the comparison between parameters according to tumor size in breast cancer tissue sample and control. The data showed insignificant differences in G0/1, S%, G2\M and G2\G1 DI, Diploidy % and Aneuploidy(P>0.05) in different breast cancer tissue samples(T1 ,T2, T3) compared with that of control benign tumor(TX).While Ki-67 showed highly significant differences in the tested groups (P=0.000).

Table (4) represents the comparison between parameters according to tumor grades in breast cancer tissue sample. The data showed insignificant differences in Ki-67, G0/1, S-phase%, G2\M, G2/G1,DI, Diploidy% and Aneuploidy (P>0.05) between GII and GIII .

Table (5) represents the comparison between parameters according to ploidy status in breast cancer tissue sample. The data showed insignificant differences in Ki-67, G2\M G2/G1between diploid and aneuploid population. While DI showed significant differences (P=0.01) and G0/1, S-phase%, DiploidY% showed highly significant differences in tested groups (P=0.005).

DISCUSION

The expression of Ki-67 protein in tumor cells is a reliable marker of proliferation in breast cancer, it has been found in G_1, S and G_2-M phases of cycling cells with increases mainly in S and G_2-M phases of the cell cycle, while it was not found in non-cycling G_0 cells. Therefore, Ki-67 is often used for measurement of proliferating tumour cells in breast cancer and its quantification may give important information on the growth characteristics of a tumor **(Gerdes, et al., 1984; Liu, et al., 2001 and Urruticoechea, et al., 2005).** The development of Ki-76 monoclonal antibody flowcytometrical analysis has given the possibility of testing this proliferative index in the routine assessment of prognostic factors. In the present study the data revealed that Ki-67 labelling index in tissue of breast cancer was significantly increased compared with the control (benign tumor). The expression of Ki-67 has been found associated with higher aggressiveness and invasiveness. **(Spyratos, et al., 2002; Neri et al., 2008 and Folkward et al. 2009)** supported our results and concluded that Ki-67 expression has a high prognostic value, when it has low values below 10% it is classified as correctly tumors with low proliferation and could be used to avoid overtreatment. In high expression of Ki-67 above 25% it is classified as highly proliferative

tumors. The median value of Ki-67 expression positivity of 15% resulted identified as the best discriminator between good and bad prognosis groups.

The present data showed that the prognostic value of Ki-67 is associated with tumor size with highly significant difference between (T1, T2, T3) in cancer tissue and Tx in benign tissue. This is confirmed by previous studies of **(Haerslev, et al., 1996 and Molino, et al., 1997)** who reported that Ki67 expression positivity was correlated with tumor size**. (Neri, et al., 2008)** concluded that Ki67 expression positivity was significantly related to larger tumor size.

In addition, the present data revealed that the prognostic value of Ki-67 is associated with histological grade, menopausal status and lymph node status. The data recorded higher values in GIII than in GII with insignificant difference between them. Ki-67 have higher values in premenopausal patients than postmenopausal with insignificant difference between them. Also Ki-67 recorded elevated value in positive lymph node than negative lymph node with insignificant difference between them.

Our results are supported by **(Neri, et al., 2008)** and reported that high Ki-67 expression was correlated to unfavorable tumor characteristics such as high tumor grade, increasing tumor size and absence of estrogen receptors resulted and identified as independent predictors of Ki67 positivity . Also found that Ki67 positivity was significantly related to premenopausal status, larger tumor size, high tumor grade and absence of estrogen and progesterone receptors. **(Folkward, et al., 2009; Spyratos, et al., 2002 and Trihia, et al., 2003)** concluded that histological grading is most clearly related to Ki-67 expression and the strongest correlation was seen between grading and Ki-67 staining. **(Molino, et al., 1997; Bader, et al., 2002 and Urruticoechea, et al., 2005)** showed that there was a strong correlation between the strongest prognostic factor in breast cancer, lymph node status. Ki-67 identified as an easy marker of nodal involvement that would avoid unnecessary axillary surgery.

In the present study, in blood1 sample obtained from cases during surgery before chemohormonal therapy, the mean of Ki-67 labelling index values of patients was significantly increased compared with controls. While in blood2 sample obtained from cases after chemohormonal therapy, Ki-67 recorded a lower value when compared with blood, but still higher than the control (benign tumor). In addition,

the present results indicated that flow cytometric evaluation of Ki-67 staining in breast cancer patients' blood cells before and after chemotherapy and hormonal therapy is a simple and effective method for predicting the effect of anticancer drugs. The postoperative adjuvant chemohormonal therapy resulted in decreasing Ki-67 expression without change in DNA ploidy pattern including the S-phase fraction. This in agreement with previous studied of **(Clarke, et a1., 1993 and Kobayashi, et al., 1995)** who concluded that the therapy of patients under investigation resulted in reducing the size of the tumor (down staging) and decreased Ki-67 expression with no change in DNA ploidy pattern including the S-phase fraction . **(Clarke, et a1., 1993)** suggested that Ki-67 would be useful for monitoring the response to antiestrogen therapy. Because hormonal treatment induces changes in Ki-67 staining but not in estrogen or progesterone receptors. Low doses of anticancer drugs may have an effect on protein synthesis and thus on Ki-67 expression but may not cause actual histological changes. **(Bundred, et al., 2002; Harper-Wynne, et al., 2002; Assersohn, et al., 2003 and Euler, et al., 2003)** reported the effective chemotherapy and mixed chemohormonal treatments in lowering Ki-67. Hormonal treatments (endocrine therapy) suppress Ki-67 levels in both short-term (< 6 weeks) and long-term (12 weeks)**.** This confirm our results that Ki-67expression in breast cancer may be a useful marker for early cell damage.

(Spyratos, 1993) described the nuclear DNA content determined by flowcytometry which used as an indicator of proliferation rate. Cellular DNA content is usually expressed as DNA ploidy of cells under investigation as ratio of aneuploid to that of normal control (diploid**)**. The measurement estimates the fraction of cells in the S phase (S-phase fraction) which expressed cells that are actively synthesizing DNA that reflects proliferative activity.

The present data showed that S-phase fraction (SPF) in tumour tissue of breast cancer was significantly increased compared with control. The elevation of SPF measurement has been found associated with higher aggressiveness and invasiveness. In blood1 samples, the SPF measurement in patients was insignificantly changed compared with the control. While in blood2 after treatment, the value of SPF significantly decreased when compared with the control. Although the prognostic value of SPF wasn`t correlated with tumour size, it is associated with histological grade, menopausal status and lymph node status. In this study SPF recorded insignificant difference between GII and GIII. In

addition highly significant value of SPF was recorded according to ploidy status.

(Sigurdsson, et al., 1990 ;Ferno, et al., 1992 and Michels, et al., 2000). agreed with the present data and reported that a high percentage of S-phase (SPF) is also indicative of a poor prognosis. SPF data may be more useful than DNA ploidy as independent prognostic predictors in cancer. **(Zhang, et al., 1997 and Jones, et al., 2001)**. showed that low SPF is associated with an excellent prognosis in breast cancer patients**. (Collan, et al., 1992 and Spyratos, et al., 1993)** supported the present data and reported that no consistent relationship has been demonstrated between SPF and tumor size and S-phase fraction based on the grading of breast cancer. **(Spyratos, et al., 1992)** indicated that DNA content and S-phase fraction measured by flowcytometry may also be useful as indicators of sensitivity to chemotherapy .

The present results indicated that G0/1, G2/M and G2/G1 have significantly increasing value in cancer compared with the control. In blood1 before any treatment, G0/1 and G2/G1 have significant values while G2/M showed insignificant value. In blood2 after response to chemohormonal therapy, G0/1 showed significant increasing value than blood1. While G2/M recorded significant decreasing value and G2/G1 illustrate insignificant decreasing value than blood1.

All of G0/1, G2/M and G2/G1 have insignificant correlation with tumor size. According to grade only G2/G1 has significant correlation with grade and both of G0/1 and G2/M have insignificant correlation with grade. Concerning ploidy status (DNA diploid versus DNA aneuploid), G2/M and G2/G1 have insignificant value while, G0/1 have highly significant value.

The present data confirmed the findings of **(Sahin, et al., 1991 and Spyratos, 1993)** who reported that the proportion of cells in S and G2M phases is higher in actively growing tumors than in slowly growing tumors ,this reflects the prognosis of cancer patients. SPF is used as an indicator for the aggressiveness (growth activity) of a tumor and indicate that DNA content measured by flowcytometry may also be useful as indicators of sensitivity to chemotherapy.

The data showed that DNA index (DI) refers to ploidy status of tumor tissue. DI showed insignificant difference ($P>0.05$) in tumor, blood1, blood2 and in correlation with other prognostic factors such as tumor size, histological grade. On other hand DI shows significant

difference value according to ploidy status. The present result suggested that the DI is considered non-independent factor in prognosis of breast cancer. Diploidy% have insignificant difference (P>0.05) in tumor, blood1, blood2 and in correlation with other prognostic factors such as tumor size, histological grade, but have highly significant difference according to ploidy status P=0.005. Aneuploidy% have significant difference (P=0.000) in tumor, blood1, blood2 but have insignificant difference in correlation with other prognostic factors such as tumor size, histological grade (P>0.05).

(Spyratos, 1993) supported the present data and concluded that the ability of DNA ploidy to provide independent prognostic information. The clinical use of DNA content measurement in breast cancer patient management has been controversial. Tumours with DNA aneuploidy and high SPF are usually more aggressive than DNA-diploid tumors with low SPF. There no consistent relationship has been demonstrated between DNA ploidy and tumor size and there is a strong association between high histological grade and both DNA aneuploidy and high SPF.

CONCLUTION

High expression in Ki-67 was significantly associated with poor prognosis in patients with women breast cancer. High Ki-67 values associated with high grade and large tumor size. This suggests that higher tumor proliferation may be important in the prognosis of breast cancer. Although FCM-DNA analysis may ultimately be useful as a prognostic indicator for breast cancer and as indicators of response to therapy by measurement of ploidy status and S-phase. This study indicated that Ki-67 is an independent prognostic factor, despite having very strong correlations with other biomarkers such as S-phase. The DNA index considered to be non-independent factor in prognosis of breast cancer. The combination of high Ki-67, SPF, G2/M, high grade and Aneuploidy with low of Go/G1 resulted in poor prognosis of patient of breast cancer. This combinatorial approach may also prove a valuable prognosis and assess response to anticancer treatment and the subsequent clinical outcomes.

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دراسة تنبوئية لسرطان الثدى في الإناث باستخدام بعض الدلائل البيولوجية

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يعتبر سرطان الثدى هو الأكثر شيوعا فى مختلف أنحاء العالم, ويمثل نسبة كبيرة من جميع حالات السرطان المسجلة فى النساء. ويتحكم حجم الورم ومرحلته ودرجته ومعدلات نموه وعوامل أخرى فى نوعية و كيفية العلاج منه. إن عدم وجود دلائل بيولوجية يمكن الاعتماد عليها فى التنبأ بمرحلته والاستجابه للعلاج كان السبب في البحث عن استحداث دلائل بيولوجية لقياس درجة تقدم المرض ومعدل نموه. وتأسيسا على ذلك فلقد تم استخدام انتجين(Ki–67) لقياس معدل انقسام الخلايا بطريقة خلوية كيميائية والتغير فى محتوى الحمض النووى (DNA) من خلال دورة الخلية فى خلايا الورم الخبيثة من الثدى وخلايا االدم البيضاء وحيدة النواة من خلال تقنية السريان الخلوى، وذلك للتنبأ بفعالية هذه الدلائل لقياس تقدم الورم ومدى استفادة المرضى من العلاج والشفاء من المرض. بينت النتائج المتحصل عليها ارتفاع معدل انقسام الخلايا(Ki–67) وتحليل الحمض النووى فى الحالات المتأخرة من المرضى والتى لم تستجب للعلاج كما يجب. كما بينت النتائج أن هذه الدلائل مجتمعة دلت على تنبأ أفضل بحالة انقسام الخلايا المرضية. وتوصى الدراسة باستخدام هذه الدلائل مجتمعة أفضل منها منفردة فى التنبؤ بحالة المرضى واستجابتهم للعلاج.