Study of serum Bcl-2, Bax and P53 proteins as biological markers in breast cancer patients

A Thesis

Presented to The Medical Research Institute
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In Partial Fulfillment of the
Requirements for the Degree

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Master

in

Applied Medical Chemistry

By

Sehame Morsy Mohamed Mohamed Rabie

B.Sc. Faculty of Science (Chemistry & Biochemistry),
Alexandria University, 2007

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List of Abbreviations

AAbs	: Autoantibodies
Abs	: Antibodies
ACD	:accidental cell death
ADP	: Adenosine 5'-diphosphate
AIF	: Apoptosis-inducing factor
APAF-1	: Apoptotic protease activating factor-1
AR	: Androgen receptor
AUC	: Area Under Curve
Bak	: B-cell lymphoma protein 2 (Bcl-2) antagonist killer 1
Bax	: B-cell lymphoma protein 2 (Bcl-2) associated X protein
Bcl-2	: B-cell lymphoma protein 2
Bcl-XL	: B-cell lymphoma protein 2 (Bcl-2) related protein, extra long isoform
BH3	: B-cell lymphoma protein 2 (Bcl-2)-homology domain 3
Bid	: Bcl-2-homology domain 3 (BH3) interacting domain death agonist
BRCA1	: Breast cancer gene 1
BRCA2	: Breast cancer gene 2
CA15.3	: Cancer antigen 15.3
CAF/FAC	: 5-Fluorouracil, adriamycin and cyclophosphamide
Caspases	: Cysteine-aspartic proteases
CBC	: Complete blood count
CICs	: Circulating immune complexes
CT	: Computed tomography
CTD	: C-terminal regulatory domain
dATP	: Deoxyadenosine triphosphate
DcR1	: Decoy receptor 1
DcR2	: Decoy receptor 2
DcR3	: Decoy receptor 3
DD	: Death domain
DED	: Death effector domain
DFI	: Disease free interval
DFS	: Disease free survival
DISC	: Death inducing signaling complex
DNA	: Deoxyribonucleic acid
DNase	: Deoxyribonuclease
DR	: death receptor
EGF	: Epidermal growth factor
EGFR	: Epidermal growth factor receptor
ELISA	: Enzyme-linked immunosorbent assay
ER	: Estrogen receptor
FADD	: Fas associated death domain
Fas, CD95	: Fatty acid synthetase, cluster of differentiation 95

FLIP/FLICE	: Fas associated death domain (FADD)-like interleukin-1β-converting enzyme (ICE)
FNAC	: Fine needle aspiration cytology
Her-2	: Human epidermal Growth Factor receptor type-2
HRT	: Hormone Replacement Therapy
IAP	: Inhibitors of Apoptosis
cIAP	: Inhibitor of Apoptosis Proteins
IGF-IR	: Insulin-like growth factor-1 receptor
LN	: Lymph node
M	: Metastasis
MAb	: Monoclonal antibody
mRNA	: Messenger ribonucleic acid
IMM	: inner mitochondrial membrane
IR	: ionizing radiation
LHRH	: luteinizing hormone - releasing hormone
LMP	: last menstrual period
MAC	: mitochondrial apoptosis- inducing channel
MDM2/HDM2	: murine/human double minute 2
Mdm2	: The murine double minute
MOMP	: mitochondrial outer membrane permeabilization
MOMP	: mitochondrial outer membrane permeabilization
MRI	: Magnetic Resonance Imaging
mTOR	: Mammalian target of rapamycin
MUC-1	: Mucin 1
NF-kB	: nuclear factor kappa-light-chain-enhancer of activated B cells
P53	: Tumor suppressor gene of Protein 53
PCD	: programmed cell death
PERP	: protein 53 apoptosis effector related to PMP-22
PI3K	: Phosphatidylinositol 3-kinase
PIG3	: protein 53- induced protein with death domain
PIDD	: protein 53-inducible gene
PBC	: Primary Breast Cancer
PR	: Progesterone receptor
R	: Spearman coefficient
ROC	: Receiver operating characteristic
ROS	: Reactive oxygen species
SE	: Standard error
Smac/Diablo	: Second mitochondria- derived activator of caspases/direct Inhibitor of Apoptosis Proteins (IAP) binding protein with low isoelectric point (pI)
T	: Tumor size
tBid	: Truncated Bcl-2-homology domain 3 (BH3) interacting domain death agonist (Bid)
TNF	: Tumor necrosis factor
TNFR	: Tumor necrosis factor receptor
TRADD	: Tumor necrosis factor receptor associated DD

TRAF2	: Tumor necrosis factor receptor associated factor 2
TRAIL	: Tumor necrosis factor related apoptosis inducing ligand
UICC	: International Union Against Cancer
UVR	: Ultra violate radiation
VDAC	: voltage-dependent anion channel

INTRODUCTION

INTRODUCTION

Cancer

Cancer can be defined as a disease in which a group of abnormal cells grow uncontrollably by disregarding the normal rules of cell division. Normal cells are constantly subject to signals that dictate whether the cell should divide, differentiate into another cell or die. Cancer cells develop a degree of autonomy from these signals, resulting in uncontrolled growth and proliferation. If this proliferation is allowed to continue and spread, it can be fatal. In fact, almost 90% of cancer-related deaths are due to tumour spreading – a process called metastasis. [1, 2]

Clonal origin of cancer

Current dogma states that cancer is a multi-gene, multi-step disease originating from a single abnormal cell (clonal origin) with an altered DNA sequence (mutation). Uncontrolled proliferation of these abnormal cells is followed by a second mutation leading to the mildly aberrant stage. Successive rounds of mutation and selective expansion of these cells (natural selection) results in the formation of a tumour mass (Figure 1). [2, 3]

Subsequent rounds of mutation and expansion leads to tumour growth and progression, which eventually breaks through the basal membrane barrier of tissues and spreads to other parts of the body (metastasis). Death as a result of cancer is due to the invading, eroding and spread of tumours into normal tissues due to uncontrolled clonal expansion of these somatic cells. [2, 4]

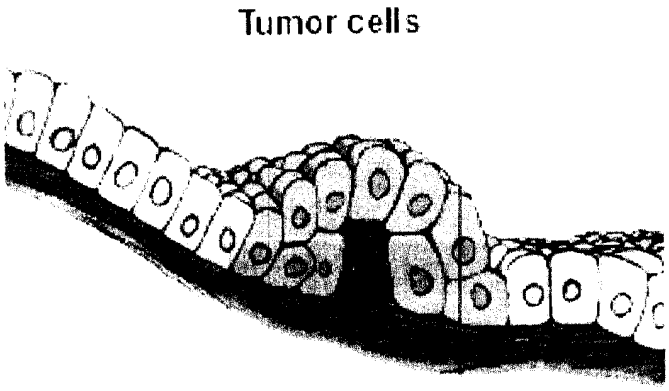

Figure 1: Clonal expansion. [2]

The main categories of cancer

Carcinoma: - cancer that begins in epithelial cells from a germ layers

Sarcoma: - cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue.

Leukemia: - cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood.

Lymphoma and myeloma: - cancers that begin in the cells of the immune system.

Central nervous system cancers: - cancers that begin in the tissues of the brain and spinal cord. [5]

Insights into cancer

Initiation and progression of cancer depends on both external factors in the environment (tobacco, chemicals, radiation and infectious organisms) and factors within the cell (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). These factors can act together or in sequence, resulting in abnormal cell behaviour and excessive proliferation. As a result, cell masses grow and expand, affecting surrounding normal tissues (such as in the brain), and can also spread to other locations in the body (metastasis). However, it is important to remember that most common cancers take months and years for these DNA mutations to accumulate and result in a detectable cancer. [6, 7]

Causes of cancer (Aetiology)

Which of the two - genes or the environment – play a dominant role in developing cancer? While genes are distributed unequally across populations, they do not explain the differences in cancer incidence rates in the world. This is demonstrated most dramatically in the following example. Incidences of stomach cancer are 6-8 times higher among Japanese compared to Americans. However, children of migrant Japanese settled in America show incidence rates of stomach cancer comparable to that of the American population. Therefore, the risk of developing cancer seems largely environmental, accounting for more than 90% of all cancers. [7, 8]

Lifestyle and Environment

The first known report linking the influence of lifestyle on cancer was linked between nasal cancer and the use of tobacco snuff. In the late 18th century, the scrotal cancer in chimney sweeps was linked to poor hygiene and accumulation of cancer-causing agents in soot. The Danish Chimney sweeper's Guild recommended daily baths and was the most likely reason for the dramatic reduction in scrotal cancer incidence rates in Europe. [9]

In 1950, compelling epidemiological evidence showed that heavy cigarette smokers ran a 20-fold higher risk of developing lung cancer compared to non-smokers. Since then, tobacco and alcohol consumption have been linked to almost ~170,000 mouth and throat cancer deaths per year in the US alone. Over half a million deaths every year are expected to be caused by lifestyle choices such as obesity, physical inactivity, diets (low in vegetables, high in salt or nitrates are linked to stomach and oesophageal cancers whereas high fat, low fibre diets are linked to bowel, pancreatic, breast and prostate cancer). [2]

Risk of cancers are also increased by infectious agents including viruses [hepatitis B virus (HBV), human papillomavirus (HPV), human immunodeficiency virus (HIV) – increase risk of hepatocellular carcinoma, cervical carcinomas and Kaposi's sarcoma] and bacteria such as Helicobacter pylori (stomach cancer). An incidence of skin cancer (melanoma) is on the rise, especially in Australia, due to exposure to high levels of ultraviolet radiation in the sun's rays and popularity of tanning salons. However the risk of developing some of these cancers can be reduced by changing lifestyles and vaccines. [10, 11]

Age

Although cancer can occur in people of every age, it is common among the aging population. Sixty percent of new cancer cases and two thirds of cancer deaths occur in persons > 65 years. The incidence of many cancers (eg, breast, colorectal, prostate, lung) increases exponentially with age. [12]

Cellular origin (histogenesis)

Determining the origin of the tumour by histopathological classification of tissue is useful in a) identifying whether the tumour is a primary or secondary tumour e.g. a liver tumour may be primary or metastasised from elsewhere or b) source of origin of the tumour e.g. lung cancer due to smoking is epithelial (lung carcinoma) but due to asbestos exposure is mesothelial (mesothelioma or asbestos cancer). [13, 14]

Shift work

Night-time shift work has been classified as 'probably carcinogenic to humans' by the International Agency for Research on Cancer (IARC). Work at night may cause breast cancer in women and could potentially cause other cancers such as prostate and endometrium. The negative health effects of shift work are thought to be related to circadian rhythm disruption, working conditions, fatigue, behavioural changes (e.g., smoking, diet), or stress. [15, 16]

The six hallmarks of cancer

DNA mutations result in defects in the regulatory circuits of a cell, which disrupt normal cell proliferation behaviour. However, individual cell behaviour is not autonomous, and cells rely on external signals from surrounding cells in the tissue or from the microenvironment. There are more than 100 distinct types of cancers and any specific organ can contain tumours of more than one subtype. Taken together, these facts raise several questions. How many of these regulatory circuits need to be broken to transform a normal cell into a cancerous one? Is there a common regulatory circuit that is broken among different types of cancers? Which of these circuits are broken inside a cell and which of these are linked to external signals from neighbouring cells in the tissue?

The answer to these questions can be summarised in a heterotypic model, manifested as the six common changes in cell physiology that results in cancer, proposed by Douglas Hanahan and Robert Weinberg. This model looks at tumours as complex tissues, in which cancer cells recruit and use normal cells in order to enhance their own survival and proliferation. The 6 hallmarks of this currently accepted model can be described using a traffic light analogy (Figure 2). [2, 17]

1) Immortality: Continuous cell division and limitless replication

2) Produce 'Go' signals (growth factors from oncogenes)

3) Override 'Stop' signals (anti-growth signals from tumour suppressor genes)

4) Resistance to cell death

5) Angiogenesis: Induction of new blood vessel growth

6) Metastasis: Spread to other sites

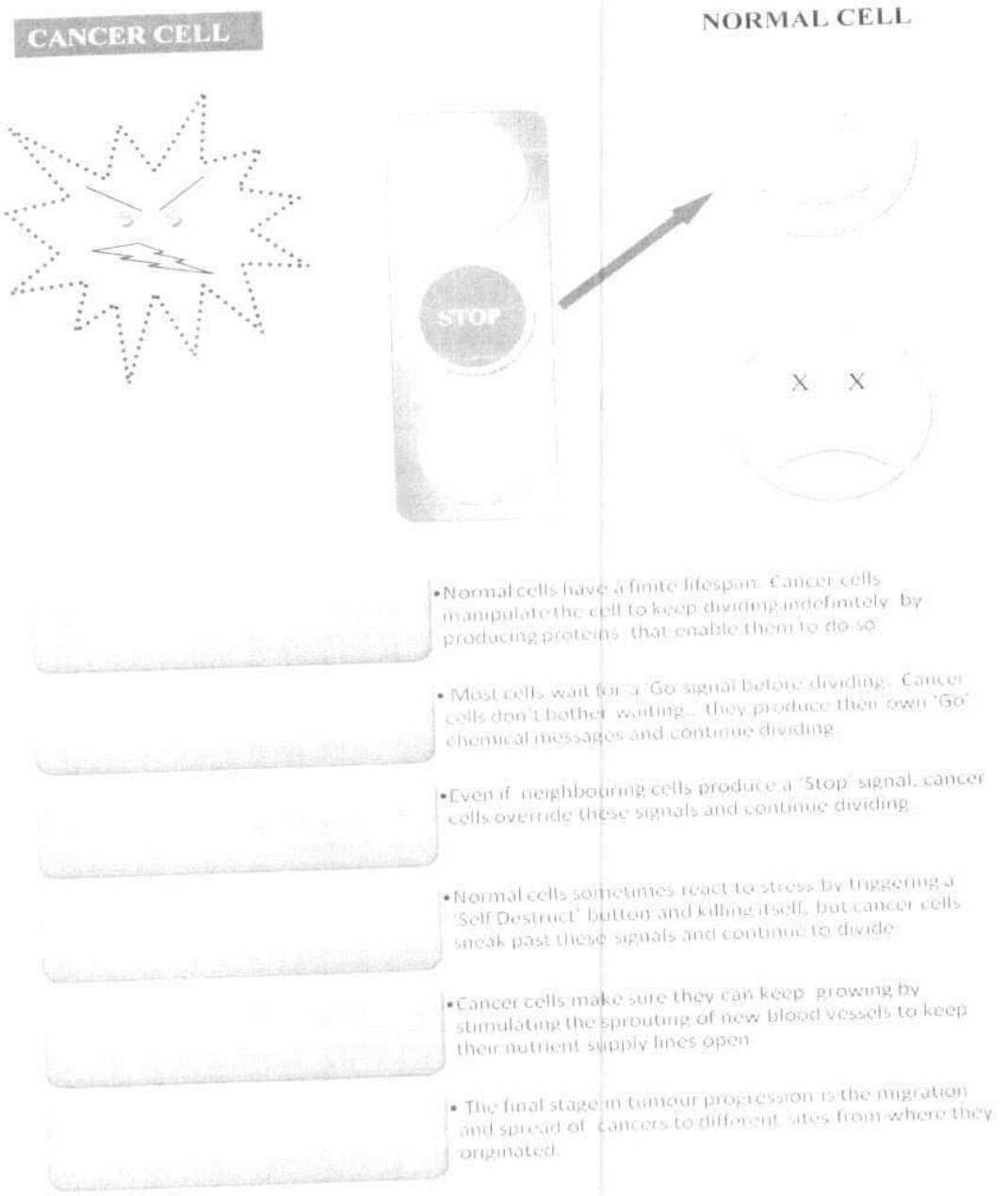

Figure 2: The six hallmarks of cancer. [2]

Breast Cancer

Incidence of breast cancer

Breast Cancer is the most common cancer in women in the UK, with a lifetime risk of 1 in 9. Approximately 44 000 women are diagnosed annually in the UK. [18]In Egypt, breast cancer is the most commen female malignancy. In Alexandria, it accounts for 42.7% of all malignancies in females. [19]

Anatomy the breast

Gross structure

The breasts are situated on either side of the sternum between the second and sixth rib, overlying the pectoralis major muscle. The shape of the breast is hemispherical with a tail of tissue extending into the axilla. The size of the breast will vary with the stage of development and age and will also vary between individuals. Centrally on each breast lies the nipple–areola complex. The nipple lies in the centre of the areola and is approximately 6 mm in length. [20]

Microscopic structure

The breast is composed of fibrous, glandular and fatty tissue and is covered by the skin. Fibrous bands divide the glandular tissue into approximately 16–20 lobes. Within each lobe is the milk-producing system. The lobe contains up to 40 lobules that contain 10–100 alveoli (or acini) which are the milk-secreting cells. The alveloi are connected to lactiferous tubules, which in turn connect to the lactiferous duct, which is lined with epithelial cells. The lactiferous duct runs up towards the nipple and, when approaching the nipple, widens to form the ampulla, which acts as a reservoir for the milk to be stored. The lactiferous duct than continues on from the ampulla to open out onto the surface of the nipple (Figure 3). The glandular tissue of the breast is surrounded by fat. [20]

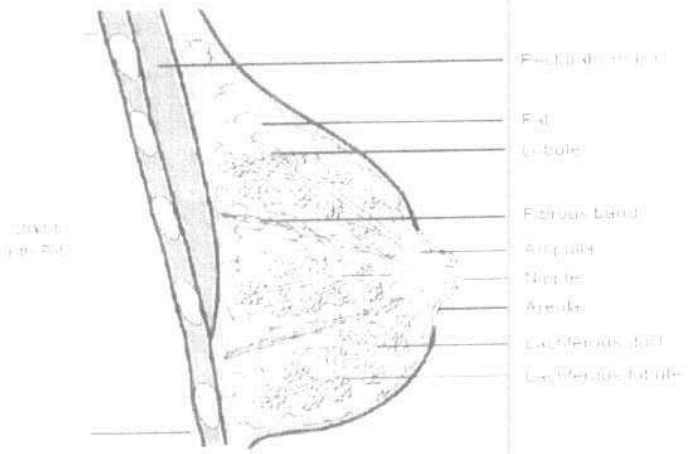

Figure 3:- Microscopic structure of the breast. [20]

Lymphatic system

The lymph fluid from the outer quadrants of each breast flows into the ipsilateral axillary lymph nodes along a chain which begins at the anterior axillary nodes and continues into the central and apical node groups. Lymph fluid from the medial quadrants drains towards the sternum via the inframammary nodes. The major lymphatic drainage of the breast is to the axilla, and the axillary nodes are the first place a breast cancer will spread to. The axillary nodes are divided into three levels (Figure 4). [21]

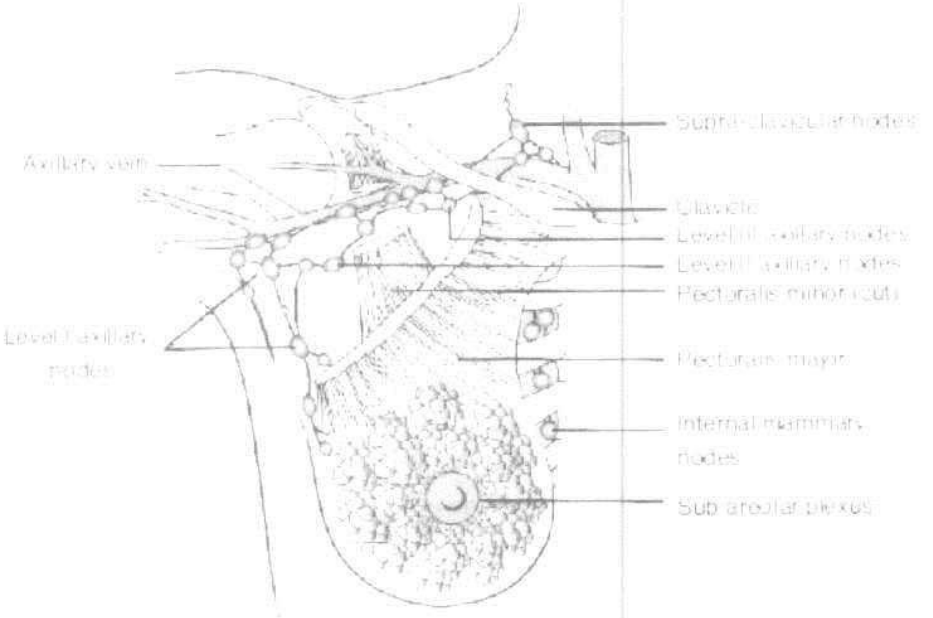

Figure 4:- Lymphatic drainage of the breast illustrating levels of axillary nodes. [21]

Risk factors

The cause of breast cancer is not yet fully understood: however some risk factors have been identified. The risk factors can be divided into two: definite risks and potential risks. [22, 23]

1) Definite risks

Definite risks are the known risks that have been shown by research to increase the risk of developing breast cancer. [24]

Gender

Being female increases the risk of breast cancer. Males do get breast cancer but it is rare. [24]

Age

As female get older, the chance of getting breast cancer increases. Breast cancer is rare in women under the age of 35 years. [25]

Strong family history

Having a strong family history of breast cancer increases the risk of developing breast cancer. Much research is being undertaken to identify faulty genes that are associated with the increased risk. Two such genes have been identified so far: BRCA1 (breast cancer type 1 susceptibility protein) and BRCA2 (Breast Cancer 2 susceptibility protein). [26]

• Note:- everyone who has a family history of breast cancer will be at a higher risk of developing breast cancer than the average population. [27-28]

Exposure to hormones

The number of menstrual cycles a woman undergoes is a powerful determinant of breast cancer risk. A woman who has an early menarche and late menopause has an increased risk of developing breast cancer. It has been shown that women who undergo a bilateral oophrectomy under the age of 50 years have a 50% reduction in breast cancer for up to 9 years post-surgery. (24) A woman who has had her first baby after the age of 35 years, or is nulliparous, also carries a higher risk of developing breast cancer. These findings suggest that the unopposed circulating oestrogen increases the breast tissue's susceptibility to other risk factors for breast cancer. [29]

Benign breast disease

Several studies have looked at the correlation between breast cancer and benign breast disease. [30]

Ionising radiation

Exposure to ionising radiation is known to increase the risk of breast cancer, as was found in studies in the use of radiation to treat benign conditions. [31-34]

Oral Contraceptive pill

The Collaborative Group on Hormonal Factors in Breast Cancer carried out a meta-analysis of results of 54 studies. It found that those who had used oral contraceptives (both combined or progesterone only) has a small but statistically significant risk that disappeared 10 years after cessation. [35] One population-base case-control study in the USA suggested that the new lower-dose pills may impart a lower risk of breast cancer than the older higher-dose preparations. [36] The combined pill also reduces the risk of ovarian cancer. [37]

Hormone Replacement Therapy (HRT)

Several studies have looked at hormone replacement therapy and its affect on breast cancer risk. [38, 39]

Obesity

Most studies have shown that obesity is a protective factor in pre-menopausal women but increases the risk in post-menopausal women. [38] This is because in post-menopausal women the major source of estrogen comes from peripheral aromatization of adrenal androgens in fat. Therefore obesity increases the risk of breast cancer. [40]

Alcohol intake

In a pooled analysis of cohort studies looking at the effect of alcohol consumption on the risk of breast cancer, Smith-Warner et al. [41] found that it is associated with an increased risk.

2) Potential risk

Potential risk is that has not been proven but has led scientists to research further. [28, 29]

Diet

As yet there is no scientific evidence to link breast cancer with diet. [26] The intake of fat in the diet has been studied showing no significant risk. Hunter et al. [42] pooled the results from eight major cohort studies and showed no effect of fat intake on the risk of breast cancer. There has been no conclusive evidence to suggest that a diet high in fruit and vegetables reduced the risk of breast cancer.

Diagnosis of breast cancer

There are two main diagnostic pathways: screening and symptomatic. The National Institute for Health and Clinical Excellence produced guidelines for referral for suspected breast cancer. By December 2009, Specialist breast units offer triple assessment which involves clinical examination, radiological assessment and pathological assessment. [43]

Methods of assessment

History taking

Before examining the patient, a detailed history should be taken. Patient's age, Past medical history, Family history of breast and ovarian cancer, Age at menarche/age at menopause, Date of the last menstrual period (LMP), Use of the combined oral contraceptive pill and hormone replacement therapy, Number of pregnancies, Age at first pregnancy, Whether the patient breast-fed her babies and Nature of the presenting symptom and its duration. [28, 29]

Clinical examination

The clinical examination is divided into two parts: [29]

1. Inspection
2. Palpation

Investigations

Following inspection and examination the clinician may organize further investigations. The following investigations are common place: [28, 29]

A) Mammography

A mammogram is a low-dose X-ray of the breast tissue. Mammograms are generally not performed on women under 35 as the breast tissue in young women is relatively radio dense. [29]

B) Ultrasound

Ultrasound is a painless procedure that uses high-frequency sound waves. It is a useful in women under 35, as an aid to mammography, measuring lesions and differentiating between a cystic or solid mass. [29]

C) Magnetic Resonance Imaging (MRI)

Breast magnetic resonance imaging is increasing used in breast assessment although it not routinely used. It is used as an addition to mammograms and ultrasound in complex cases. [28, 29]

D) Fine-needle aspiration cytology

Fine-needle aspiration cytology (FNAC) can be performed freehand by the clinician in the outpatient department or under ultrasound guidance. [29, 44]

E) Core Biopsy

If the FNAC results are not conclusive, or histology is required, a core biopsy can be taken. Increasingly the use of core biopsy is replacing FNAC. [29]

Staging investigations

If breast cancer is diagnosed, staging investigations may be undertaken to assess for metastatic disease. A chest X-ray, a bone scan, abdominal ultrasound, computed tomography (CT) or MRI (Magnetic Resonance Imaging) can be requested. [28, 29]

Staging breast cancer

The pathologist is responsible for reporting the histological findings. The results should be discussed at a multidisciplinary team meeting. [29]

The pathologist will report of different characteristics of the tumour including:

1) Size

The size of the tumour is one of the most significant prognostic indicators. The smaller the cancer is, the better the prognosis. [28]

2) Grade

The tumour is graded according to the cellular differentiation i.e. the degree to which the cancer cells resemble their tissue of origin. [28] A commonly used grading system is the modified Bloom and Richardson system. It uses three grades: Grade I, Grade II and Grade III [29]

3) Vascular and lymphatic invasion

If the tumour has invaded the blood or lymphatic vessels this is a poor prognostic feature. [28]

4) Lymph node status

The number of lymph nodes involved with cancer cells determines the chance of survival for that individual and is one of the most important prognostic indicators. [29]

5) Hormone Receptor status

The tumour is analysed to test for the presence of the steroid hormone receptors, oestrogen (ER) and progesterone (PR). The presence of such receptors will determine the effectiveness of endocrine therapy such as tamoxifen and the aromatase inhibitors. It has been shown that those with an oestrogen receptor positive tumour have a better outcome. [28]

6) Oncogenes

Changes to the genes in a normal cell can result in cell proliferation and malignant proliferation. Proto-oncogenes are involved in stimulating the cell through the normal cell cycle resulting in proliferation, while tumour suppressor genes inhibit excessive cell proliferation. However, either mutation or amplification of proto-oncogenes, and inactivation or loss of tumour suppressor genes can result in uncontrolled cell proliferation and cancer formation. Many proto-oncogenes encode for epidermal growth factor receptors, e.g. human epidermal growth factor receptor - 1 (Her-1) and the human epidermal growth factor receptor-2 (Her-2). [29, 45]

Stage classification

Staging refers to the grouping of patients according to the extent of their disease. The purpose of this grouping is as follows: To aid the clinician in the planning of treatment; to

give some indication of prognosis and to assist in the evaluation of the results of treatment. [28, 29]

Several classification systems are in use, most commonly the UICC (International Union Against Cancer) TNM system and the Nottingham Prognostic Indicator. [28, 29]

The TNM system

The TNM system was developed in France between 1943 and 1952 and is used for all tumour types, not solely breast. The TNM system is based on three main components: T – the extent of the primary tumour, N – the absence or presence and extent of regional lymph node metastasis and M – the absence or presence of distant metastasis (table 1). [46]

Table 1:- Clinical stage grouping [46]

Stage	TNM classification		
0	Tis	N0	M0
I	T1	N0	M0
IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
IIIB	T4	Any N	M0
IIIC	Any T	N3	M0

Treatment of breast cancer

A. Surgical treatment

1) breast conservation therapy

It involves the total gross removal of tumor by limited surgery followed by Radiotherapy to eradicate any residual tumor left in the remaining breast tissue. An axillary node dissection or sentinel node procedure should be done for staging purposes. [47]

2) Modified radical mastectomy

It is the standard surgical procedure. This procedure includes complete removal of the breast as well as axillary lymph node resection. [48]

B. Radiation therapy

Radiation therapy is treatment with high - energy rays or particles that destroy cancer cell. This treatment may be used to kill any cancer cells that remain in the breast, chest wall or underarm area after breast-conserving surgery. Radiation may also be needed after mastectomy in patients with either a cancer larger than 5 cm in size, or when cancer is found in the lymph nodes. [49]

C. Chemotherapy

Chemotherapy is treatment with cancer-killing drugs that may be given intravenously or by mouth. The drugs travel through the bloodstream to reach cancer cells in most parts of the body. It is given in cycles and usually lasts for several months. [28, 29]

There are several situations in which chemotherapy may be recommended:

Adjuvant chemotherapy:

It is given to patients with no evidence of cancer after surgery. Surgery is used to remove all of the cancer that can be seen, while the goal of adjuvant chemotherapy is to kill undetected cells that have traveled from the breast. [29, 50, 51]

Neoadjuvant chemotherapy:

It is chemotherapy given before surgery. It uses the same drugs that are used as adjuvant therapy (only it is given before surgery instead of after). The major benefit of neoadjuvant chemotherapy is that it can shrink large cancers enough to be removed by lumpectomy instead of mastectomy. [28, 29]

D. Endocrine therapy

1) Selective ER modifiers:

Tamoxifen has been considered the standard of care for all women with an invasive breast cancer that expresses either ER or PR. Trials have demonstrated that patients did the best when taking 20 mg of Tamoxifen daily for 5 years. [28, 29, 52]

2) Aromatase inhibitors:

Block the peripheral conversion of the adrenal androgens (androstenedione and testosterone) into estradiol and estrone in women. Aromatase inhibitors should not be considered in those women who have any ovarian function. [28, 29, 53]

3) Ovarian ablation:

Ovarian ablation via surgical oophorectomy, or suppression with agonists of luteinizing hormone - releasing hormone (LHRH) are effective therapy for premenopausal ER-positive early stage. [28, 29]

Prognostic markers of breast cancer:

Today, prognosis is determined by a number of clinical and biological parameters currently including lymph node status, tumor size, grade of malignancy, age, absence or presence of vascular invasion and Her-2 gene expression. Using these parameters, patients are allocated to one of three risk groups (low, intermediate and high risk, respectively) and adjuvant therapy is planned according to this. However, it is becoming increasingly clear that the currently available prognostic parameters are relatively inadequate to precisely define the prognosis of individual patients. Thus, additional prognostic markers need to be identified (Table 2). [54]

Table 2: Biomarkers used in breast cancer treatment [54]

Class/pathway	Biomarker	Abbreviation	Therapeutic Opportunity
Nuclear receptor pathways	Estrogen receptor-alpha	ER	Selective ER modulators (tamoxifen) Histone deacetylase(HDAC) inhibitors (vorinostat)
	Progesterone receptor	PR	Progesterone antagonists
	Androgen receptor	AR	Androgen hormone
Growth factor receptor pathways	Epidermal growth factor receptor	EGFR	Abs, TKIs (gefitinib, lapatinib) Abs (trastuzumab),TKIs,HER2 intracellular domain (ICD) peptide-based vaccine
	Epidermal growth factor receptor type -2	HER-2	
	insulin-like growth factor-1 receptor	IGF-1R	Abs, TKIs
DNA repair pathways	Breast cancer gene 1 and 2	BRCA1/2	Poly(ADP)-ribose polymerase 1(PARP1) inhibitors, interstrand crosslinks-generating drugs (mitomycin-c, cis-platinum, and its analogues), lovastatin
Phosphatidylinositol 3-kinase (PI3K) pathway	Mammalian target of rapamycin	mTOR	mTOR inhibitors(rapamycin, CCI-799)

Abs, antibodies; TKIs, tyrosine kinase inhibitors.

Apoptosis

Cell proliferation, differentiation and death are fundamental processes in multicellular organisms, and several lines of evidence link apoptosis (programmed cell death, PCD) to proliferation. Many physiological processes, including development and homeostasis, require a balance between apoptosis and cell proliferation. [55] If this equilibrium is disturbed, the cells will either divide faster than they can die, resulting in cancer development or die faster than they can divide, resulting in tissue atrophy. [56] Apoptosis and cell proliferation are linked by cell-cycle regulators and apoptotic stimuli that affect both processes. [57]

Apoptosis occurs in several normal and pathological processes among which are the elimination of excess of cells during the embryogenesis, the elimination of cells after the suppression of the stimulus, the clonal selection of lymphocytes during the induction of autotolerance, the elimination of cells in tissues with a great cellular change over, the elimination of infected cells by viruses (by T cytotoxic lymphocytes), the elimination of cells with DNA alteration, elimination of neoplasic cells, cell death in neurodegenerative diseases (Alzheimer) and hypoxia. There are four main events which cause the apoptosis (Figure 5). [58]

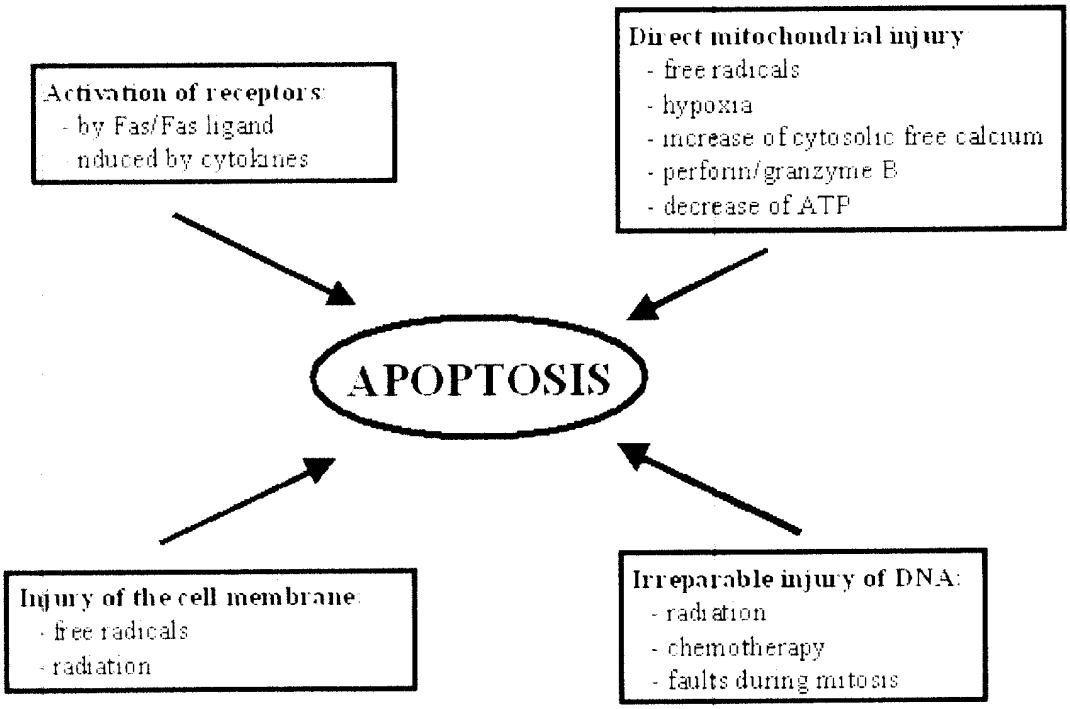

Figure 5:- Main triggers of apoptosis pathways. [58]

Control of the apoptotic mechanisms

The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly (extrinsic inducers) or intracellularly (intrinsic inducers). Extracellular signals may include toxins, hormones, growth factors, nitric oxide or cytokines, that must either cross the plasma membrane or transduce to effect a response. These signals may positively (i.e., trigger) or negatively (i.e., inhibit) affect apoptosis. Binding and subsequent trigger of apoptosis by a molecule is termed positive induction, whereas the active repression or inhibition of apoptosis by a molecule is termed negative induction. [59, 60]

Mechanisms of Apoptosis

The mechanisms of apoptosis are highly complex and involving an energy-dependent cascade of molecular events. There are two main apoptotic pathways: the extrinsic (death receptor pathway) and the intrinsic (mitochondrial pathway). However, there is now evidence that the two pathways are linked and that molecules in one pathway can influence the other. There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A. (Figure 6). [61-63]

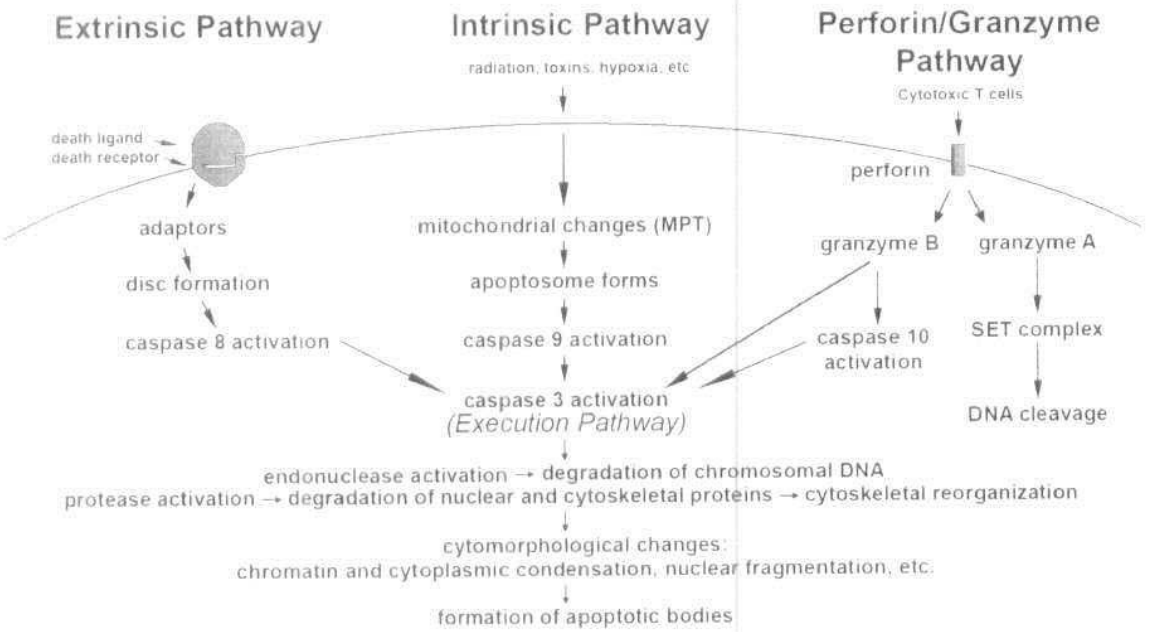

Figure 6:- Schematic representation of apoptotic events. [63]

There are four stages of the apoptotic process. The main characteristics of which are (Figure 7): [58]

I- Induction/signalling phase

Where molecular systems are activated by: Bcl-2 (B cell lymphoma) family proteins as Bax (Bcl-2 associated X protein). This stage is absence of morphologic changes. It is reversible stage. [58]

II- Effector phase

It is no return point. Loss of mitochondrial transmembrane potential that causes the "opening" of a large ionic channel called "permeability transition pore complex or megachannel" through which mitochondrial substances are released into the cytosol: cytochrome C, Smac/Diablo, endonuclease G, AIF. The megachannel in the mitochondrial membranes leads to depolarization, mitochondrial swelling, ATP depletion and cell death (Figure 8). [64]

III- Degradation phase

Several enzymatic mechanisms are activated including effectors caspases 3, 6 and 7. Crosslink among proteins are broken, DNA degradation, phosphatidyl serine is exhibited in the outer cell membrane. Morphological changes appear: cell surface specializations and cell-cell junctions are broken, chromatin is condensed and the endonucleases fragment the chromatin into separated nucleosomes, subsequently the nucleus is fragmented and the cell breaks into several fragments (apoptotic bodies). This process lasts only a few minutes, organelles are undamaged. [58]

IV- Phagocytic phase

The phosphatidyl serine position in the outer cell membrane provides the recognition by macrophage phagocytosis. Absence of inflammatory response even after exposure to typical apoptotic stimuli, apoptosis can be switched to necrosis when ATP levels are reduced. Thus, ATP level is undoubtedly one of the key factors that determine the cell death fate. [64, 65]

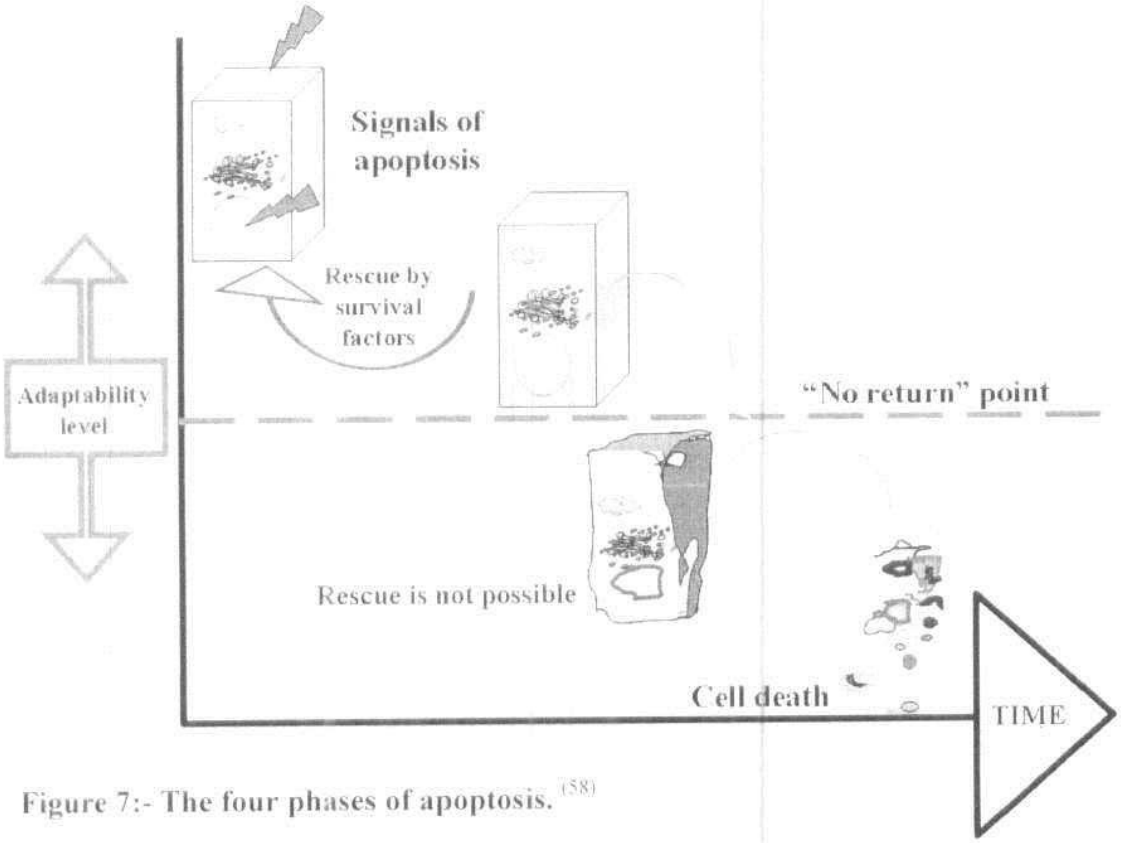

Figure 7:- The four phases of apoptosis. [58]

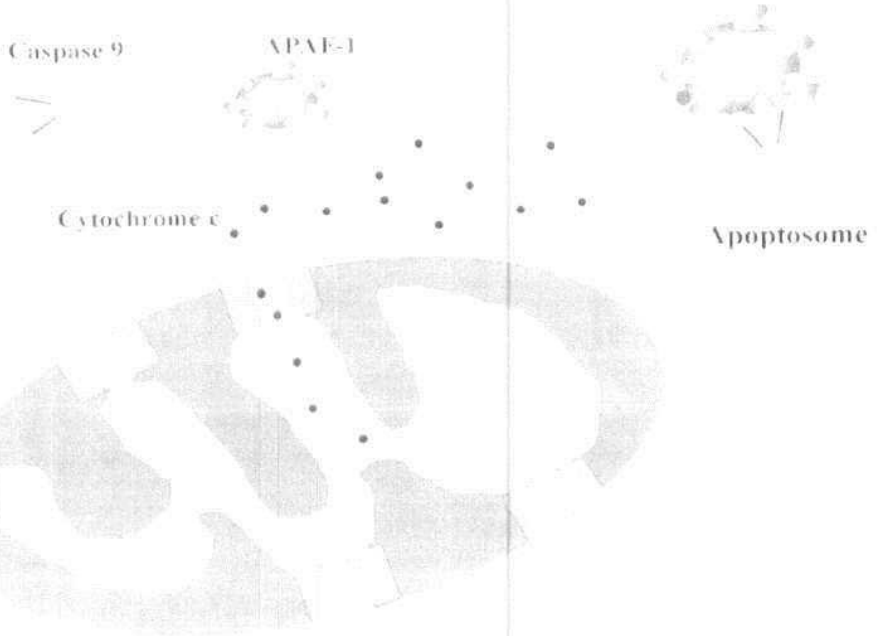

Figure 8:- The opening of the permeability transition pore complex. [64]

Extrinsic pathway of apoptosis

The TNF family acts by binding the extracellular domains of receptor proteins. Each member of the TNF family binds to one or more receptors in the TNFR family and some receptors bind one or more ligands. Ligand binding elicits a multitude of responses including apoptosis, proliferation and inflammation and the given response depends upon the adapter proteins the bound receptor recruits. [66, 67]

Soluble TNF family ligands TNFR apoptosis-inducing ligand (TRAIL), FasL, and TNF form trimers that recognize and bind their cognate death receptors. I) FasL and TRAIL: after binding receptors, DR4, DR5, and Fas undergo conformational changes resulting in assembly of the death inducing signaling complex (DISC). Decoy receptor 1 (DcR1), DcR2, and DcR3 bind ligand with high affinity but do not induce apoptosis. DR4, DR5, and Fas then recruit Fas-associated death domain (FADD) through complementary death domains (DDs). FADD can recruit caspase 8 through their complementary death-effector domains (DEDs). Recruitment of caspases 8 to the DISC leads to its autoproteolytic cleavage, releasing two subunits that form active enzyme. In type I cells, caspase 8 cleaves and sufficiently activates effector caspases 3, 6, and 7 to fully engage the apoptotic response. In type II cells, activated caspase 8 cleaves Bid, which stimulates Bax and Bak to release factors from the mitochondria, including cytochrome c, thus activating the intrinsic pathway of apoptosis. II) TNF: TNF binds TNF-R1 and recruits TNFR-associated DD (TRADD) through its DD and a complex of proteins containing receptor-interacting protein (RIP) and TNFR-associated factor 2 (TRAF2) (Complex I). Complex I can activate inhibitor of nuclear factor (NF)-κB (IκB) kinase complex, thereby freeing NF-κB for entry into the nucleus and rapid transcription of anti-apoptotic genes, including FLICE inhibitory protein (FLIP) and cIAP1/2. [68] Complex I then dissociates from TNF-R1 where it binds FADD and caspase 8 (Complex II). If in sufficient abundance, FLIP can block Complex II's caspase 8 from self-activation. Otherwise, complex II triggers a caspase 8-driven apoptotic response (Figure 9). [68]

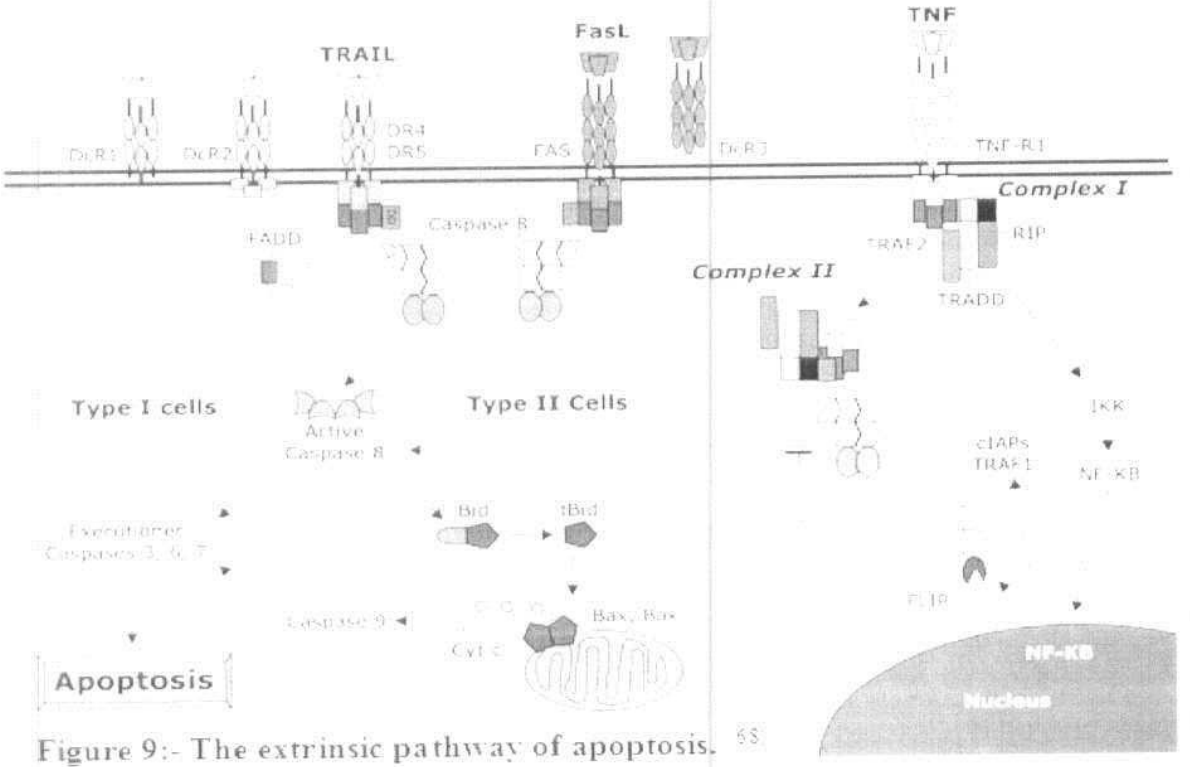

Figure 9:- The extrinsic pathway of apoptosis. [65]

The Intrinsic Pathway of Apoptosis

The mitochondrial or intrinsic pathway appears to play a predominant role in the apoptotic response to anticancer drugs. Bcl-2 family members regulate this pathway by modulating the release of key proapoptotic polypeptides, including cytochrome c and second mitochondrial activator of caspases (Smac)/direct inhibitor of apoptosis (IAP)-binding protein with low PI (DIABLO), from mitochondria. Several Bcl-2 family members that facilitate mitochondrial permeabilization are transcriptional targets of the p53 tumor suppressor gene, providing a partial explanation for the ability of DNA-damaging agents to induce apoptosis (Figure 10). [69]

The intrinsic pathway integrates a broad spectrum of extracellular and intracellular stresses. The intracellular stimuli include DNA damage, oxidative stress with production of reactive oxygen species (ROS), endoplasmic reticulum stress, growth factor deprivation as well as the mitochondrial dysfunction and extracellular stimuli including starvation, radiation and cytotoxic drugs. Mitochondrion is a central organelle in the intrinsic apoptotic pathway. Its dysfunction and release of mitochondrial proteins to the cytosol initiates the apoptotic cascade. [70] Upon stress, intrinsic pathway is triggered via activation of pro-apoptotic members of Bcl-2 family proteins (Bax, Bak), which oligomerise on the outer mitochondrial membrane and cause mitochondrial dysfunction. The proapoptotic action of Bax and Bak can be antagonised by the antiapoptotic members of the same family Bcl-2 or Bcl-XL. Following mitochondrial dysfunction, several apoptogenic factors, including cytochrome c and (SMAC/DIABLO), are released from the mitochondrial intermembrane space into the cytosol. [71]

The intrinsic pathway involves transduction of various signals into a change in permeability of the outer mitochondrial membrane, which then leads to activation of caspase 9. A critical step in this pathway is the selective release of polypeptides from the mitochondrial intermembrane space into the cytoplasm. The most widely studied of these released polypeptides is cytochrome c. Release of the cytochrome c from mitochondria during apoptosis appears to be rapid. Upon its appearance in the cytoplasm, cytochrome c binds the scaffolding molecule apaf-1, which then undergoes a nucleoside triphosphate dependent conformational change and binds procaspase 9. The resulting complex termed the "apoptosome" contains multiple apaf-1 and procaspase 9 molecules, binding to this complex activates procaspase 9, which then proteolytically activates caspase 3. [72] Once activated, caspase 3 selectively cleaves several hundred substrates, and caspase 6, which is activated by caspase 3, cleaves a smaller group of polypeptides. These cleavages contribute too many of the changes observed during apoptosis. Caspase-induced cleavages also participate in the biochemical changes observed in apoptotic cells (Figure 10). [73]

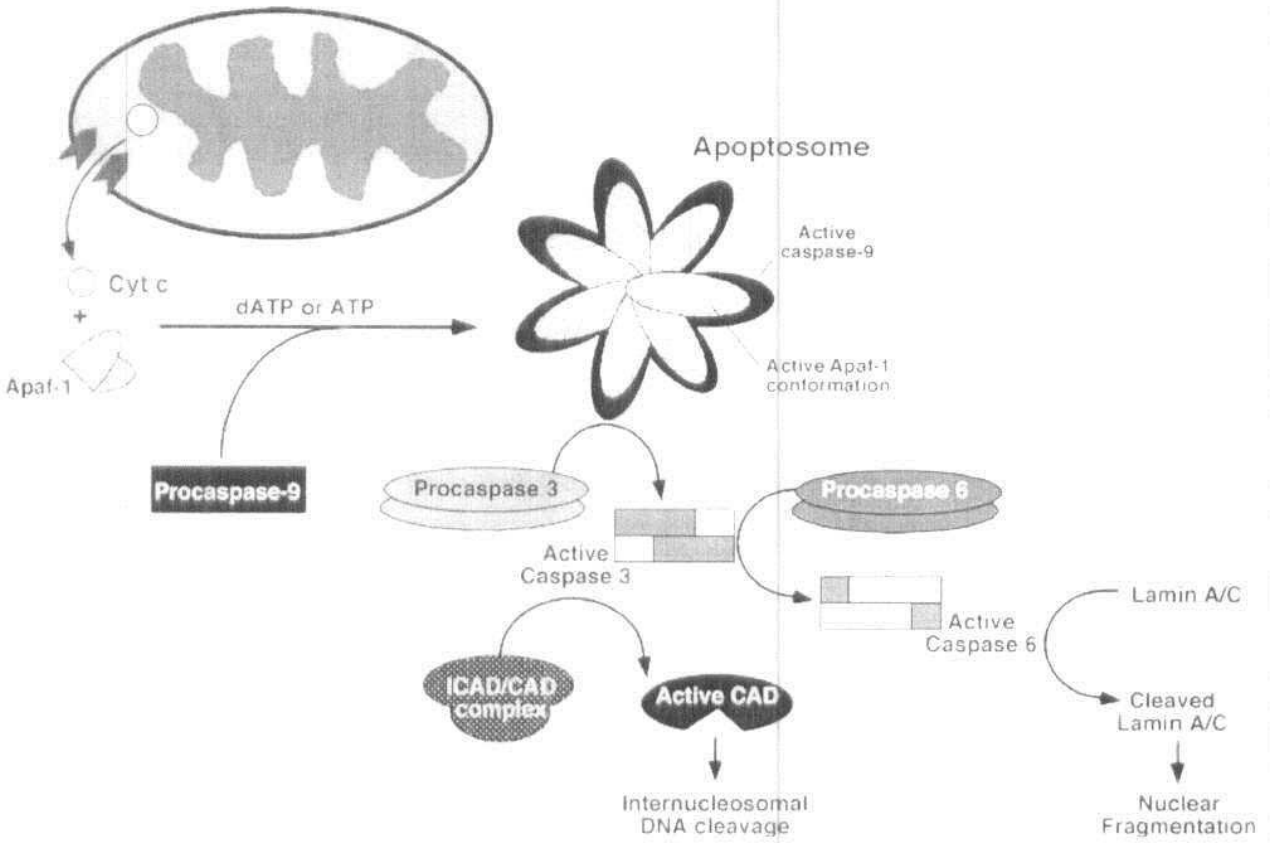

Figure 10:- The intrinsic (mitochondrial) pathway of apoptosis. [69]

Defective apoptotic pathways

The many different types of apoptotic pathways contain a multitude of different biochemical components, many of them not yet understood. As a pathway is more or less sequential in nature, it is a victim of causality: removing or modifying one component leads to an effect in another. In a living organism this can have disastrous effects, often in the form of disease or disorder. A discussion of every disease caused by modification of the various apoptotic pathways would be impractical, but the concept overlying each one is the same: the normal functioning of the pathway has been disrupted in such a way as to impair the ability of the cell to undergo normal apoptosis. This results in a cell that is able to replicate and pass on any faulty machinery to its progeny, increasing the likelihood of the cell becoming cancerous or diseased. [74]

Tumour resistance to apoptosis

The life and death of cells must be balanced if tissue homeostasis is to be maintained. Too much growth and too little death can lead to a severe disturbance that might result in cancer. In multicellular organisms, many of the mechanisms that control tissue homeostasis are linked to apoptosis. Defects in the apoptosis-inducing pathways can eventually lead to expansion of a population of neoplastic cells. Resistance to apoptosis can also augment the escape of tumour cells from surveillance by the immune system. Moreover, because chemotherapy and irradiation act primarily by inducing apoptosis, defects in the apoptotic pathway can make cancer cells resistant to therapy. So, resistance to apoptosis constitutes an important clinical problem. [75]

Every cell in a multicellular organism has the potential to die by apoptosis, but tumour cells often have faulty apoptotic pathways. These defects not only increase tumour mass, but also render the tumour resistant to therapy. [76]

Cancer treatment by chemotherapy and gamma-irradiation kills target cells primarily by inducing apoptosis. Therefore, modulation of the key elements of apoptosis signaling directly influences therapy-induced tumour-cell death. Tumour cells can acquire resistance to apoptosis by the expression of anti-apoptotic proteins or by the downregulation or mutation of pro-apoptotic proteins. [76]

How can we kill cancer cells?

Induction of apoptosis by chemotherapeutics is considered as one of the major anti-cancer effects leading to inhibition of tumour growth. Over the past years our understanding of signaling pathways associated with induction of apoptosis, and knowledge on executioners of apoptosis, has substantially increased. Recently, cell-to-cell stochastic variability has become central to apoptotic cell death signaling. [77, 78]

Bcl-2 family

The Bcl-2 family of proteins belongs to a class of proteins regulating apoptosis, cell cycle, differentiation, and autophagy. In oncology, the genes coding for these proteins could not be defined neither as dominant transforming oncogenes (such as myc), nor tumor suppressor genes (such as p53 [Protein 53]). They could be best defined as apoptosis-related genes.[79]

The Bcl-2 family Features [80]

1) The Bcl-2 family of proteins includes both pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) molecules. Indeed, the ratio between these two subsets helps determine the susceptibility of cells to a death signal.

2) Their frequent ability to form homo- as well as heterodimers.

3) Their ability to become integral membrane proteins.

The Bcl-2 family proteins functions

1) Apoptosis

Several Bcl-2 family are proteins involved in programmed cell death in most tissues including mitochondria. Both antiapoptotic and proapoptotic Bcl-2 family proteins have C-terminal transmembrane domains that are inserted in the outer membrane of mitochondria. Proapoptotic Bcl-2–family proteins, such as Bax and Bak, induce mitochondrial outer membrane permeabilization (MOMP), causing the release of caspase activating proteins and other cell death mediators, whereas antiapoptotic proteins such as Bcl-2 serve as guardians of the outer membrane and preserve its integrity by opposing Bax. Other nonmitochondrial pathways for apoptotic cell death also exist, including those governed by tumor necrosis factor-family death receptors, such as Fas receptor. However, even the death receptor pathway ("extrinsic pathway") converges with the mitochondrial pathway ("intrinsic pathway") in certain types of cells, through caspase-mediated cleavage and activation of Bid, an endogenous modulator of Bcl-2/Bax–family proteins (Figure 11).[81, 82]

2) Necrosis

Bcl-2–family proteins are also mediators of necrotic cell death, e.g., defects in electron transport chain in respiring mitochondria releasing ROS into cells, causing lipid peroxidation and membrane damage, which impair normal ion homeostasis, causing cellular swelling and plasma membrane rupture, as well as rupture of lysosomes and release of hydrolytic enzymes that destroy proteins, nucleic acids, and lipids. The point of regulation may be linked to the ability of Bcl-2–family proteins to control outer mitochondrial membrane permeability of ROS (Figure 11).[82, 83]

3) Autophagy

Bcl-2 suppress autophagy by binding the protein Beclin, an essential component of the mammalian autophagy system that marks autophagic vesicles for fusion with lysosomes for digestion and recycling of components. The antiautophagic function of Bcl-2 has been dissociated from mitochondrial location, and instead appears to be manifested from the endoplasmic reticulum (Figure 11). [82, 84]

At the molecular level, control of apoptosis as well as control of cell cycle, differentiation and autophagy, occur through a complex process of protein-protein interaction. In the inhibition of apoptosis this process involves heterodimerization, especially with the pro-apoptotic member of the Bcl-2 family. [85]

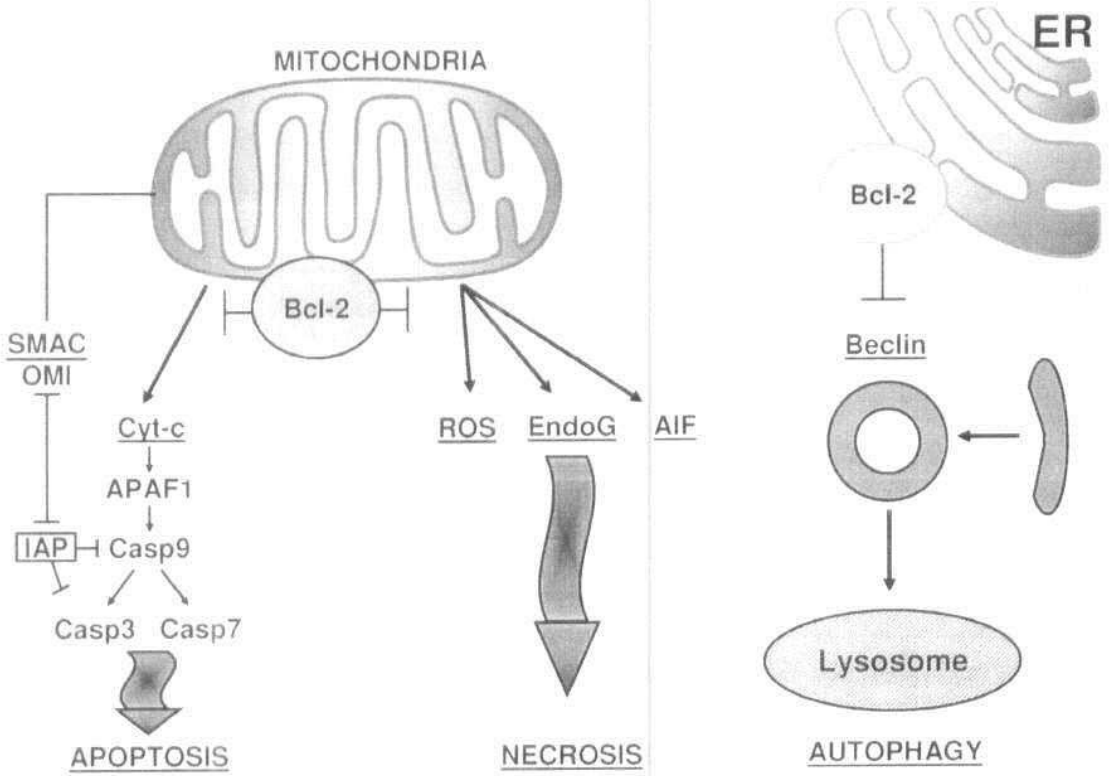

Figure 11:- Bcl-2–family proteins govern cellular pathways involved in apoptosis, necrosis and autophagy. [82]

Bcl-2 protein

Bcl-2 was first identified through its involvement in B-cell lymphomas. The bcl-2 gene codes for a 25-26 kDa protein. The C-terminal 21 amino acids encode a stretch of hydrophobic amino acids that are important for their functions where required for insertion into outer mitochondrial membranes. It has been demonstrated that insertion into membranes is closely associated with the ability of Bcl-2 to regulate apoptosis. [86]

Bcl-2 protein function

1. A function as membrane channels for ions and proteins

Bcl-2 protein is localized to the outer mitochondrial membrane, the endoplasmic reticulum and the intracellular membrane of the nuclear envelope. In these areas they have a membrane transport function for calcium ions and proteins. The ability to form channels is essential for Bcl-2 anti-apoptotic function. These channels might have different transport selectivity or subcellular localization. The channels formed by Bcl-2 and the other anti-apoptotic members prevent apoptosis, possibly transporting back, and thus antagonizing, the pro-apoptotic factors that outflow through the channels formed by the pro-apoptotic members of the Bcl-2 family. [86, 87]

2. A function as membrane adaptor/docking proteins

Bcl-2 family proteins interact with a number of signal transducing proteins involved in apoptosis and other crucial cellular processes. These include the protein kinase C homologue Raf-1, the P53-binding protein (P53-BP2) and the pro-apoptotic protein CED-4. The association between Bcl-2 and these proteins might be responsible for their translocation to intracellular membranes where Bcl-2 is anchored. This may lead to changes of their activity, such that they might be sequestered and inactivated, or targeted for interaction with other membrane associated proteins. [87]

Bcl-2 may also act as a modulator of response to chemotherapy and/or endocrine therapy where it might cause delayed onset of cell death rather than complete suppression. Bcl-2 had been shown to be expressed in many tissues, including more than half of breast cancer tissues, although, on the contrary, it was associated with favorable prognostic factors and better survival. [88]

Role of Bcl-2 protein in cell cycle control and tumorigenesis

The role of Bcl-2 antiproliferative function is complex. Bcl-2 expression reduces both proliferation and apoptosis early in the process. Association of Bcl-2 with differentiated phenotypes and better prognosis is borne out in human breast cancer studies. These solid tumors are characterized by a proliferative pretumor phase during which Bcl-2 antiproliferative effect could be more consequential than its antiapoptosis activity. Therefore, the balance between the antiapoptotic and the cell cycle effects of Bcl-2 can be influenced by tumor physiology. Bcl-2 protein, which is expressed in fetal tissues and basal cells of human epithelia, has a role in normal growth regulation and differentiation. [89, 90]

Bax protein

Bax is a protein of the Bcl-2 gene family. It promotes apoptosis by competing with Bcl-2 protein. The Bax was the first identified pro-apoptotic member of the Bcl-2 protein family. In healthy mammalian cells, the majority of Bax is found in the cytosol, but upon initiation of apoptotic signaling, Bax undergoes a conformation shift, and inserts into organelle membranes, primarily the outer mitochondrial membrane. The expression of Bax is upregulated by the tumor suppressor protein P53, and Bax has been shown to be involved in p53-mediated apoptosis. [91]

Bax function

Bax is pro-apoptotic member in Bcl-2 family. Bax promotes apoptosis, induces permeabilization of the mitochondrial outer membrane, controls transition of small molecules through mitochondrial membrane and maintains endoplasmic reticulum Ca^{2+} levels. [92-94]

Mechanism of Bax activation and function

A. Bax exists as a latent globular protein already present in the cytosol. The folded structure hides a hydrophobic cleft that is partially composed of the BH3 homology domain (green box), and a putative C-terminal domain, which has structural similarity to a mitochondrial targeting sequence (red box). The process of Bax activation begins with a change in protein conformation, which exposes the hydrophobic cleft, the N-terminus, and the mitochondrial targeting sequence. Recent studies indicate that this interaction is not directly with the Bax BH3 domain (Figure 12). [95, 96]

B. Bax becomes integrated into the MOM. Generally, proteins with these domains appear to be recognized by chaperones that facilitate movement of the protein to the MOM. Studies on other proteins containing mitochondrial targeting sequences, suggested that this domain interacts with some chaperone that helps direct these proteins to the appropriate organelles. Currently, there is no direct evidence that Bax interacts with a cellular chaperone (Figure 12). [96, 97]

C. Activated Bax appears to have the ability to recruit and activate other Bax molecules without the requirements of interactions with separate BH3-only peptides, or a chaperone to direct translocation. Once some Bax proteins become inserted into the MOM, they appear to act as a sink for other Bax molecules. As Bax molecules aggregate in the MOM, they potentially create pore structures that facilitate the release of cytochrome c and downstream events of the apoptotic cascade (Figure 12). [96, 98]

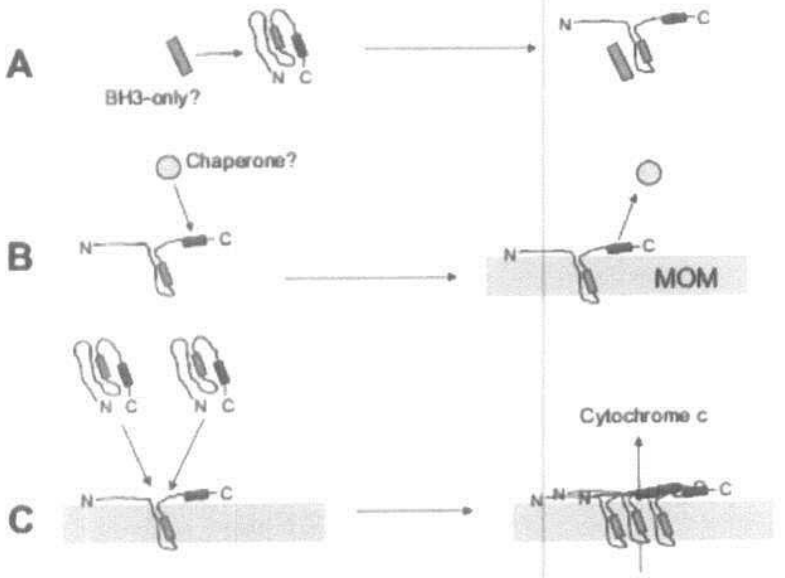

Figure 12:- Mechanism of Bax activation and function. [96]

The effect of Bax expression on tumorigenesis

Whereas Bcl-2 is prosurvival and antiproliferative, bax is proapoptotic and proliferative, suggesting that the absence of bax may promote G0. If Bcl-2 is oncogenic, then bax might be expected to be tumor suppressive. Yet, bax deficiency alone or in combination with p53 deletion was not oncogenic, bax deficiency did enhance transformation, presumably by blocking apoptosis, resulting in further enhancement of proliferation. The proliferative effect of bax was presumably dominant over its proapoptotic activity. Thus, the proapoptotic function of bax is tumor suppressive and its proliferative function is oncogenic. The relative contribution of each function to the overall effect appears to be influenced by the choice of its oncogene partners. [89, 99]

The interplay between Bax and Bcl-2

The interplay between proapoptotic and antiapoptotic proteins is part of the normal regulation of apoptosis in healthy cells. Under non apoptotic conditions the equilibrium between the cytosolic and mitochondrial pools of Bax is maintained by its Bcl-2 dependent retrotrans location. During apoptosis this balance may be modified increase the insertion of Bax into the MOM. (Figure13). [100, 101]

The presence of antiapoptotic proteins in the MOM is required to constitutively retrotranslocate Bax into the cytosol. Notably, the rate of retrotranslocation is almost doubled in cells overexpressing Bcl-2 and requires the physical interaction between Bcl-2 and Bax. Once in the cytosol, Bax quickly returns to its monomeric form, ready to cycle back to the mitochondrial surface. [101, 102]

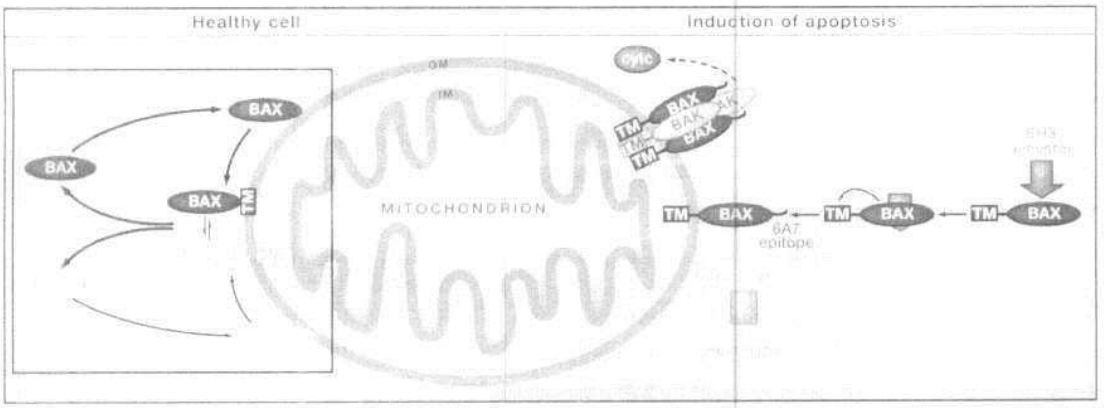

Figure 13:- Bax movements in healthy cells and in presence of apoptosis. [100]

Protein 53

P53 (also known as protein 53 or tumor protein 53), is a tumor suppressor protein. P53 is crucial in multicellular organisms. Cell cycle tumor suppressor cancer p53 has been described as "the guardian of the genome", the "guardian angel gene", and the "master watchman", referring to its role in conserving stability by preventing genome mutation. The name p53 is in reference to its apparent molecular mass: it runs as a 53-kilodalton (kDa).[103, 104]

P53 Structure

Human p53 consists of 393 amino acids, with 5 proposed domains as follows (Figure 14).[105, 106]

1) The transactivation domain (TAD; amino-acid residues 1–42), required for transcriptional activation.

2) The proline-rich domain (PRD; residues 61–94), containing 5 PXXP motifs (where P is a proline and X any other residue) that enable protein–protein interactions. This domain is thought to participate in the regulation of p53 stability and activity.

3) The DNA-binding domain (DBD; residues 102–292) specifically binds to DNA recognition elements in the promoters of target genes.

4) The tetramerization domain (4D; residues 324–355) is essential for the activity of p53.

5) The C-terminal regulatory domain (CTD; residues 363–393) that binds DNA nonspecifically and might regulate specific DNA binding by the DBD.

P53 pathway during the cell cycle

In a normal cell the protein level of p53 is under the tight control of its negative regulator murine/human double minute 2 (MDM2/HDM2) via ubiquitination. Upon DNA damage or other stresses, various pathways will lead to the dissociation of the p53 and mdm2 complex. Once activated, p53 will induce a cell cycle arrest to allow either repair or survival of the cell or apoptosis to discard the damaged cell. How p53 makes this choice is unknown (Figure 15).[107, 108]

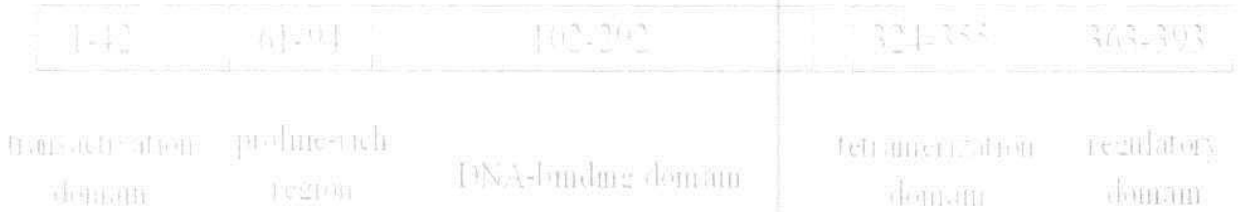

Figure 14: Schematic representation of the p53 structure. [105, 106]

NLS: nuclear localization signal sequence. NES: nuclear export signal sequence.

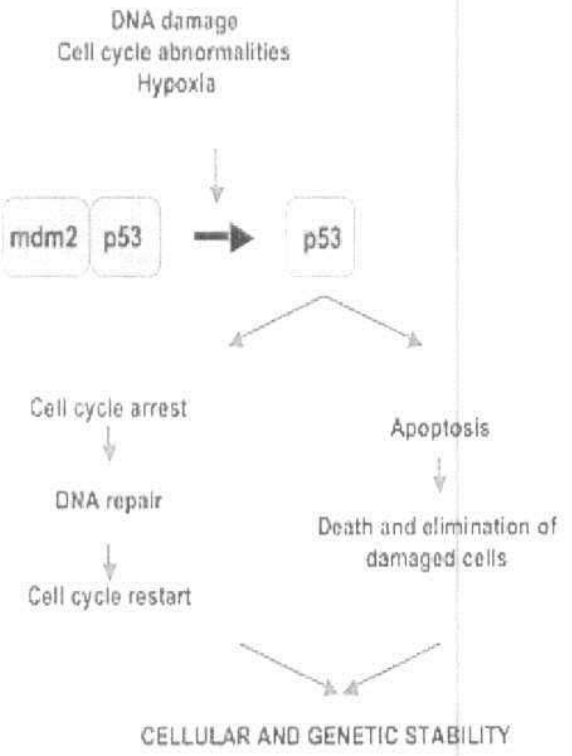

Figure 15:- p53 pathway during cell cycle. [107]

The physiological functions of p53

P53 is a poly-functional protein which functions in the nucleus. The p53 gene is continuously transcribed and translated, but the protein is rapidly subjected to ubiquitin dependent degradation in proteosomes. Therefore, the concentrations of p53 in cells of most tissues are quite low and may be at the limit of detection. The activation of p53 in response to various stresses and damages proceeds mainly post-translationally via a decrease in its degradation rate and a change of its conformation to generate increased functional activity. [109 110]

P53 works as a tumor suppressor, it is essential for preventing inappropriate cell proliferation and maintaining genome integrity following genotoxic stress. Following various intracellular and extracellular stimuli, such as DNA damage (by means including ionizing radiation, UV radiation, application of cytotoxic drugs or chemotherapeutic agents, and infectious virus), heat shock, hypoxia, and Oncogenes over expression, p53 is activated and emerges as a pivotal regulatory protein triggering diverse biological responses, both at the level of a single cell as well as in the whole organism. [111]

Many of the multiple functions of p53 including its role in tumor suppression, can be attributed to its ability to act as a sequence-specific transcription factor which regulates expression of different cellular genes to modulate various cellular processes. However, in response to various types of stress, p53 is accumulated in the nucleus and binds to specific sites in the regulatory regions of p53- responsive genes, then strongly promotes the transcription of such genes. [112]

Various intercellular or extracellular stresses elicit cellular responses directly or indirectly through p53 activation. P53 activates its downstream targets to perform various functions including cell cycle arrest, DNA repair, apoptosis, and senescence (Figure 16). [113]

Cell-cycle regulation

Among various cellular responses induced by p53, the most notable are the induction of cell cycle arrest and apoptosis. It appears that the ability of p53 to prevent cell growth is pivotal to its tumor suppressor functions. P53 can induce cell cycle arrest in the G1, G2 and S phases of the cell cycle. The induction of cell cycle arrest at G1 and G2 by p53 provides additional time for the cell to repair genomic damage before entering the critical stages of DNA synthesis and mitosis. The arrested cells can be released back into the proliferating pool through p53's biochemical functions that facilitate DNA repair including nucleotide excision repair and base excision repair. [114]

Induction of apoptosis

P53 works as a cellular gatekeeper to monitor cellular stress and to induce apoptosis as necessary. In tissues where stressors generate severe and irrevocable damage, p53 can initiate apoptosis, there by eliminating damaged cells. [115]

Apoptotic gene products which are induced by p53 include Bax, DR5/KILLER (death receptor 5), Fas/CD95 (cell-death signaling receptor), PIG3 (p53-inducible gene 3), Puma (p53-upregulated modulator of apoptosis), Noxa (from the Latin word for "harm" or "damage"), PIDD (p53- induced protein with death domain), PERP (p53 apoptosis effector related to PMP-22), Apaf-1 (apoptotic protease- activating factor-1), Scotin, p53AIP1 (p53- regulated apoptosis-inducing protein 1), and others. The products of these genes may induce apoptosis through either an extrinsic or intrinsic pathway (Figure 17). [112]

1. The intrinsic apoptotic pathway

The intrinsic apoptotic pathway is engaged when cells are challenged by stress and is dominated by the Bcl-2 family proteins. [116] In the regulation of the intrinsic pathway, pro-apoptotic gene products such as Bax, Bid, Puma, Noxa, and p53AIP1 localize to the mitochondria and promote the loss of mitochondrial membrane potential and release of cytochrome c, resulting in the formation of the apoptosome complex with Apaf-1 and caspase 9 (Figure 17). [112, 117]

cytoplasmic p53 can induce apoptosis by interacting directly with Bcl-2 family proteins which reside at the mitochondrial membrane. For example p53 activates the proapoptotic protein Bax to promote the permeabilization of the outer-membrane of the mitochondria. Although this pathway is independent of transcription, nevertheless, mice deficient in PUMA (the proapoptotic gene upregulated by p53) are resistant to DNA damage-induced apoptosis. It appears that p53 also associates with Bcl-2 which normally prevents p53 from activating Bax. A peptide derived from the BH3 domain of PUMA disables this complex thus leading to apoptosis. This suggests that after DNA damage, p53 accumulates both in the nucleus and in the cytoplasm, the latter binding to Bcl-2. Upon the induction of PUMA by the nuclear p53, the p53-Bcl-2 complex falls apart and thus Bax is activated. These studies parallel new insights that are emerging into the role of mitochondria in cell metabolism and revealing differences between cancerous and non-cancerous cells that may be exploited for therapy. Further development of new drugs and targets in this area is expected over the next few years. [118-120]

2. The extrinsic apoptotic pathway

Another p53-related class of proapoptotic gene products is the components of the death receptor-mediated extrinsic pathway. In this cell death pathway, p53 can promote apoptosis through activation of the death receptors located at the plasma membrane, including Fas/CD95, DR4 and DR5, and lead to inhibition of the production of IAPs (inhibitor of apoptosis proteins). Both DR5 and DR4 can induce apoptosis by TRAIL (tumor necrosis factorrelated apoptosis-inducing ligand), Fas ligand and chemotherapeutic agents (Figure 17). [112, 121] P53 may also induce apoptosis via an endoplasmic reticulum-dependent mechanism by transactivating the expression of Scotin, a protein located in the endoplasmic reticulum and in the nuclear membrane. [122]

The ability of p53 to induce apoptosis appears to be well correlated with its ability to suppress malignant transformation. Loss of p53-dependent apoptosis accelerates with tumorigenesis. These results reveal that regulation of apoptosis is an important and evolutionarily conserved tumor suppressor function of p53. [112, 123]

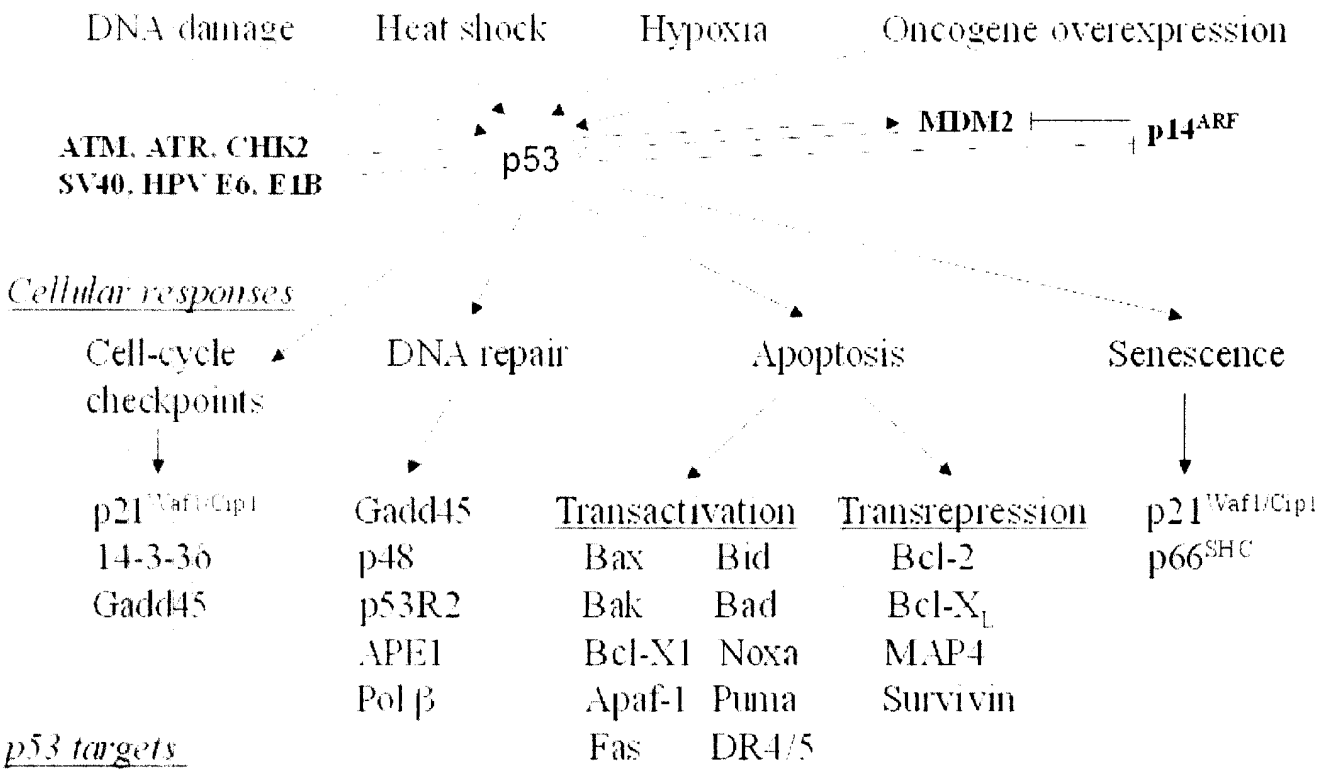

Figure 16: P53 locating at the crossroads of complex networks of stress response pathways. [113]

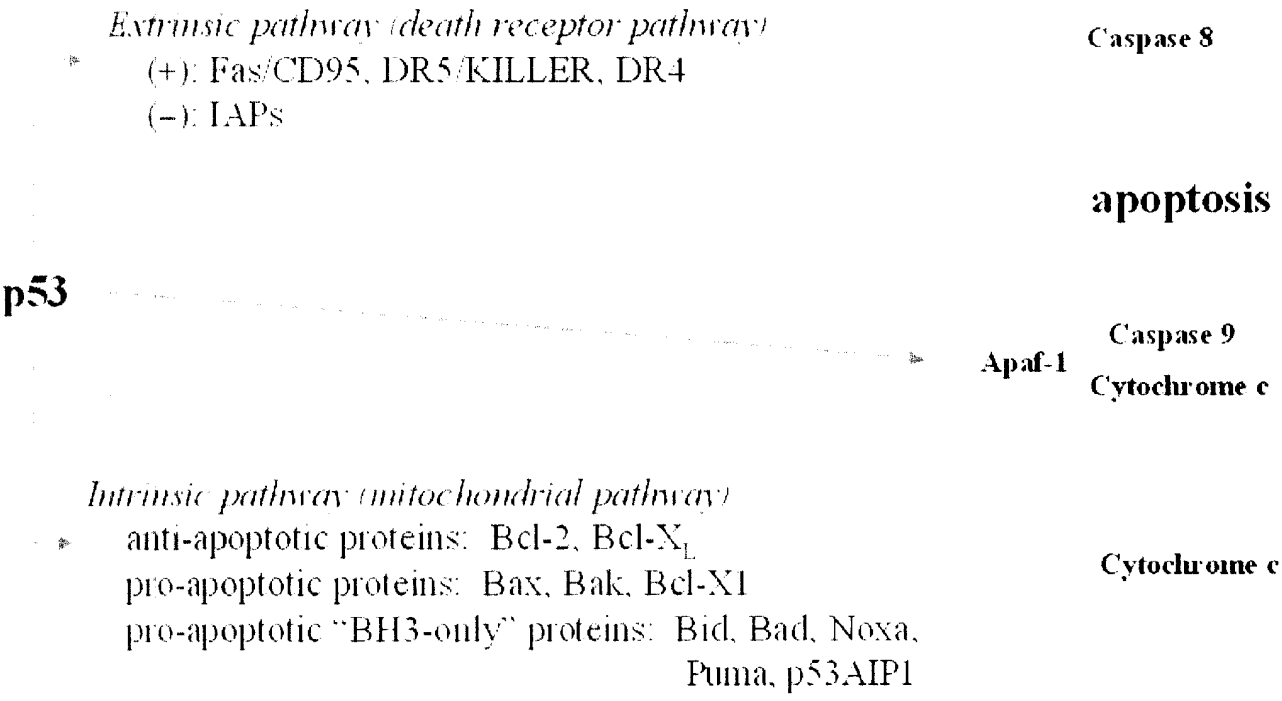

Figure 17: P53 associated genes and pathways involved in apoptotic cell death. [112]

Tumor markers

Tumor markers are substances that can be found in the body when cancer is present. The classic tumor marker is a protein that can be found in the blood in increased levels when a certain type of cancer is present, but not all tumor markers are like that. Some are found in urine or other body fluid, and others are found in tumors and other tissue. Traditionally, tumor markers are divided into two broad categories: (1) tumor-derived markers – these are molecules produced by the neoplastic cells; and (2) tumor-associated or host-response markers – these include metabolic and immunologic products of normal tissue produced in response to the presence of neoplastic tissue. Most tumor markers are proteins, but some newer markers are genes or other substances. [124]

There are many different tumor markers. Some are seen only in a single type of cancer, while others can be found in many types of cancer. The marker is usually found by combining the blood or urine with manmade antibodies that react with the tumor marker protein. Sometimes a piece of the tumor itself is tested for tumor markers. [125]

Tumor markers alone are rarely enough to show that cancer is present. Most tumor markers can be made by normal cells as well as by cancer cells. Sometimes, noncancerous diseases can also cause levels of certain tumor markers to be higher than normal and not every person with cancer may have higher levels of a tumor marker. This is why only a few tumor markers are commonly used as a reference. The level of a certain tumor marker along with the patient's history and physical examination and other laboratory tests or imaging tests can indicate the patient's status. In recent years, newer types of tumor markers are developed. With advances in technology, levels of certain genetic materials (DNA or RNA) can now be measured. It's been hard to identify single substances that provide useful information, but now looking at patterns of genes or proteins in the blood have begun. [126-130]

Uses of tumor markers

Tumor markers can be used in a number of ways as follows:

a) Screening

Screening tests require high sensitivity to detect early-stage disease. No tumour marker has yet demonstrated a survival benefit in randomized controlled trials of screening in the general population. [131]

b) Disease staging

For determining diagnosis and prognosis. [132]

c) Assessing response to therapy

Tumour marker values returning to normal may indicate cure despite radiographic evidence of persistent disease. In this circumstance, the residual tumour is often non-viable. [133]

Conversely, tumour marker levels may rise after effective treatment (possibly related to cell lysis), but the increase may not necessarily mean treatment failure. However, a consistent increase in tumour marker levels, coupled with lack of clinical improvement, may indicate treatment failure. [133]Residual elevation after definitive treatment usually indicates persistent disease.

Following tumour marker response is particularly useful when other evidence of disease is not readily accessible. [134]

d) **Monitoring for cancer recurrence**

When monitoring these patients, tumour marker levels should be determined only when there is a potential for meaningful treatment. [133]

Specific tumor markers

Tumor markers can assist in determining if a cancer is present; they can assist in determining the source of widespread cancer when the origin of the cancer is unknown. By using the information that these markers can provide, patient-specific treatment protocols can be developed, implemented, and monitored for improved patient outcomes. Example of the most commonly-used tumor markers is CA 15.3 or MUC1. [135]

Cancer antigen 15.3 (CA 15.3)

CA 15.3, also known as MUC1, episialin, polymorphic epithelial mucin (PEM) or epithelial membrane antigen, is a glycoprotein that is found in the epithelium of apical surfaces of many wet organs such as bladder, breast, colon, respiratory tract and pancreas. [136-140]

CA 15.3 structure and origin

MUC1-mucin is a high molecular weight transmembrane glycoprotein that exceeds 400KD. It is the encoded product of the MUC1 gene, which is localized to chromosome 1q21.q24. (140) The protein part of this molecule consists of a tandem repeat section consisting of 20 amino acids, to which O-linked carbohydrates are attached. [141] The protein has a variable number of these 20 amino acid tandem repeats. [142]

MUC1 protrudes out quite substantially from the cell surface due to the carbohydrate side chains that maintain an extended confirmation. Such variations result in a highly variable molecule attained from the MUC1 gene, which, in normal subjects, is found on the apical aspect of epithelial cells in organs such as the breast, lung, colon and benign ovarian tumors. [136, 138, 143, 144]

MUC1 is either secreted or shed and can therefore be found in the serum of normal healthy individuals but in low amounts. Its physiological role may be in cell adhesion and cell signaling. MUC1 may also be involved in binding to pathogens and therefore aids protection from these pathogens. [145]

CA 15.3 in carcinogenesis

Mucin genes encode rod-shaped apomucin cores that then undergo post-translational modification in the cytoplasm by glycosylation thus resulting in the MUC1 glycoprotein. During carcinogenesis, glycosylation of MUC1 is altered due to enzyme deficiencies or increased activity, resulting in an abnormal MUC1, which may then be shed into the lymph and blood circulation. Their presence in this environment allows the monitoring of carcinoma patients. [146]

Due to the wide spectrum of MUC1 in different types of normal healthy cells and thus the vast numbers of different carcinomas which express altered forms of MUC1, many immunoassays employing different monoclonal antibodies are available to detect this rise in MUC1. Measurement of MUC1 using the serum CA15.3 assay is now the most widely used serum marker assay for the monitoring of breast cancer, where it can be used to determine metastatic breast cancer recurrence as well as monitoring therapy in those with advanced breast cancer. [146-149] Immunohistochemical staining using anti MUC1 monoclonal antibodies such as CT2 MAb and C595 MAb showed that almost all breast cancer cells are stained mainly in the cytoplasm (93% of cells) and the cell membrane (73% of cells). [150]

CA 15.3 and cancer prognosis

Presence of MUC1 in either tissue or serum has been used to ascertain prognosis of breast cancer by many authors. Clearly there is no collective agreement on its prognostic value although more recent papers indicate a poorer disease outcome. Differences in prognosis may be due to the different methods of MUC1 detection as well as differences in study design.

The staining pattern itself can also correlate with prognosis. One study showed a worse prognosis and reduced disease free survival in those with greater than 75% of cells expressing MUC1, especially where the MUC1 staining was cytoplasmic. [151] In one study, the staining pattern was confirmed to be mostly cytoplasmic (93% of cells). MUC1 staining was also noted in the apical membrane in 15% and circumferential membrane in 13%, although the majority of tumour cells appear to have a mixed pattern. This compares to only apical membrane staining in normal and benign cells. This study confirmed a poorer prognosis with cytoplasmic staining. [152]

MUC1 antigens were prevalent in non-invasive breast cancer. In this group of early breast cancers, tumours with cytoplasmic staining were more commonly high grade and therefore it was concluded that cytoplasmic MUC1 was an early development in breast carcinogenesis. [153] It has been postulated that since MUC1 is suggested to function in cell adhesion, inappropriate expression during carcinogenesis could result in altered adhesion and therefore aid metastasis. [154]

Serum CA 15.3 levels in normal & cancer conditions

Serum CA 15.3 is the most widely measured serum tumour antigen in breast cancer. The CA 15.3 antigen however can also be measured in the blood of healthy women. Serum CA 15.3 is also elevated in physiological and non-malignant states such as pregnancy, lactation and infective conditions. However pregnancy and ovarian cancer-derived MUC1 is morphologically different to MUC1 from normal tissues. Interestingly, this difference in MUC1 structure is also seen in MUC1 derived from pleural and peritoneal effusions. [154-157]

Measuring serum MUC1 is mainly used for the detection of metastatic recurrence of breast cancer and also monitoring therapy in advanced breast cancer. Elevated serum MUC1 levels are also noted in other types of cancer e.g. ovarian and pancreatic cancers although no clinical use is established in these cancers. [146-161]

Serum MUC1 does not correlate with tissue MUC1 expression. Its measurement in the serum is dynamic and will correspond to therapy. The expression of antigens in the primary tumour did not correlate with which antigen became elevated in serum at diagnosis of metastases in the same individuals. [162,163]

Serum MUC1can be measured in between 5% - 30% of preoperative breast cancers according to various studies.This variance may be dependent on stage of the disease. Robertson and colleagues noted only an 8% prevalence of MUC1 in the serum of women with primary Breast Cancer (PBC) in stage I and II. This rose to 33% when women with stage III cancer were analyzed. The variations in sensitivities in different studies may be due to the various assays used to measure MUC1 antigens in the serum. [164-166]

Some authors have reported that a raised level of MUC1 in the serum indicates a poorer prognosis. A persistent rise in serum MUC1 levels above cut-off value following primary chemotherapy for locally advanced breast cancer further indicates reduced disease free interval (DFI) in this group of patients. One study noted that both tumour stage and preoperative CA15.3 can independently predict survival in PBC patients. [168-170]A large study on lymph node (LN) negative breast tumors also highlighted a poorer prognosis with patients who have increasing serum levels of MUC1 In the same study there is no correlation with tumour size, grade, stage and ER status and serum MUC1 levels. [171]

MUC1 derived from breast cancer is structurally different from MUC1 derived from normal cells and as a result the former appears to be recognized as "non-self" and induces an immune reaction to this molecule, which can be detected either as free Autoantibodies (AAbs) to various epitopes in the MUC1 molecule or as MUC1-bound circulating immune complexes (CICs). [172]

Normal MUC1 protein has these epitopes hidden from the host's immune system by heavily glycosylated side chains. However, shortened glycan side chains as found in breast cancer, result in the unmasking of these immunodominant regions. These then appear to induce an immune response. Another suggested mechanism for the development of an immune response to MUC1 is its prolonged exposure to the immune system in breast cancer patients. [173, 174]

AIM OF THE WORK

AIM OF THE WORK

The aim of this work was to investigate the diagnostic, prognostic and follow up roles of serum Bcl-2, Bax and P53 proteins in breast cancer patients in comparison with those of serum CA15.3 as the most commonly used breast cancer marker.

SUBJECTS AND METHODS

SUBJECTS AND METHODS

Subjects:

This study included 60 females divided into 2 groups:

Group I: Included 30 females having breast carcinoma of clinical stages II or III. All patients were recently detected, not receiving chemotherapy. Patients selected from those admitted to Experimental and Clinical Surgery Department and Cancer Management and Research Department, Medical Research Institute, University of Alexandria, In the period from February 2009 to February 2011

Group II: Included 30 normal healthy females of matched age, menstrual state and socioeconomic status as the previous group, volunteers clinically free from any disease (control group).

Exclusion criteria: all females suffering from immunological, liver diseases, other malignancies, history of chemotherapy or radiotherapy were excluded from this study.

All Patients met the following criteria:

- Having primary invasive breast carcinoma.
- No Clinical manifestation of infection.
- Not receiving immunomodulatory agent in recent 3 weeks.
- Not receiving blood transfusion in recent 3 weeks.

Methods:

To all patients the following were done:

- Full history recording and clinical examination.
- Routine laboratory investigations including complete blood count (CBC), bleeding and coagulation times.
- Mammography of breast and ultrasonography of abdomen.
- Radiological investigations including x-rays chest, CT scan and bone scan when needed.
- Pre-operative fine needle aspiration cytology (FNAC) of the breast mass to establish the pathological diagnosis.

All patients had undergone modified radical mastectomy. The data of tumor size, lymph node involvement, estrogen receptor (ER), progesterone receptor (PR) status, pathological grade and clinical stages were collected. All patients received intravenous adjuvant combination chemotherapy consisting of (FAC) 5-Fluorouracil, Adriamycin and cyclophosphamide and then repeated every 21 day for 6 cycles. The patients were re-evaluated after 6 cycles of chemotherapy clinically laboratory and radiologically to estimate clinical response. All patients will be followed up for 2 years.

Blood sampling:

The study design involved collecting venous blood samples in different phases of disease management and follow up. For every patient, the first sample was taken prior to operation, the second post-operatively one month after surgery before chemotherapy and the third sample was taken after 6 cycles of chemotherapy. One sample was taken from normal healthy females of control group. Venous blood samples were taken and allowed to clot thoroughly for 30 minutes before centrifugation, centrifuged at 3,000 rpm for 10 minutes and stored at -80 °C until assayed. In each blood sample, Bcl-2, Bax, P53 measured by ELISA kits while CA15.3 estimated by IRMA kit.

Determination of serum Bcl-2 levels

Bcl-2 was measured by commercially available ELISA kit for the quantitative detection of human Bcl-2. Bender MedSystems GmbH Campus Vienna. Austria. Europe.[186]

Principles of the Assay:

1. An anti-human Bcl-2 coating antibody is adsorbed onto microwells (Figure 18 a).

Figure 18a

2. Human Bcl-2 present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human Bcl-2 antibody is added and binds to human Bcl-2 captured by the first antibody (Figure 18b).

Figure 18b

3. Following incubation unbound biotin-conjugated anti-human Bcl-2 antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human Bcl-2 antibody (Figure 18c).

Figure18c

4. Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells (Figure 18d).

Figure 18d

5. A colored product is formed in proportion to the amount of human Bcl-2 present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human Bcl-2 standard dilutions and human Bcl-2 sample concentration determined (Figure18e).

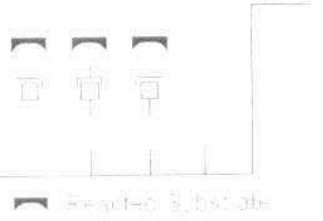

Figure 18e

Reagents Provided

- 1 aluminium pouch with a Microwell Plate coated with monoclonal antibody to human Bcl-2
- 1 vial (100 µl) Biotin-Conjugate anti-human Bcl-2 monoclonal antibody
- 1 vial (150 µl) Streptavidin-HRP
- 2 vials human Bcl-2 Standard lyophilized, 64 ng/ml upon reconstitution
- 1 vial (12 ml) Sample Diluent
- 1 vial (5 ml) Assay Buffer Concentrate 20x
  (PBS with 1% Tween 20 and 10% BSA)
- 1 bottle (50 ml) Wash Buffer Concentrate 20x
  (PBS with 1% Tween 20)
- 1 vial (15 ml) Substrate Solution (tetramethyl-benzidine)
- 1 vial (15 ml) Stop Solution (1M Phosphoric acid)
- 1 vial (0.4 ml) Green-Dye
- 1 vial (0.4 ml) Blue-Dye
- 1 vial (0.4 ml) Red-Dye
- 4 Adhesive Films

Preparation of Reagents

Buffer Concentrates were brought to room temperature and were diluted before starting the test procedure.

1. 50 ml of the Wash Buffer Concentrate (20x) were brought to a final volume of 1000 ml graduate cylinder and bought to a final volume of 1000 ml with distilled water.

2. 5 ml of the Assay Buffer Concentrate (20x) were poured into a clean 100 ml graduate cylinder and brought to a final volume of 100 ml with distilled water.

3. 1:100 dilution of the concentrated Biotin-Conjugate was prepared by add 60 µl of Biotin-Conjugate with 5.94 ml of assay buffer (1x) in a clean plastic tube.

4. 1:100 dilution of the concentrated Streptavidin-HRP solution by add 120 µl of Streptavidin-HRP solution with 11.880 ml (1x) assay buffer in a clean plastic tube.

5. Human Bcl-2 Standard was reconstituted by addition of distilled water then mixed gently to insure complete and homogeneous solubilization. (Concentration of reconstituted standard = 64 ng/ml). Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

Test Protocol

1. Each sample, standard, blank and optional control sample should be assayed in duplicate.
2. Microwell strips were washed twice with approximately 400 µl Wash Buffer per well with thorough aspiration of microwell contents between washes. Wash Buffer was allowed to sit in the wells for about 10 – 15 seconds before aspiration.
3. After the last wash step, wells were emptied and microwell strips were tapped on absorbent paper to remove excess Wash Buffer.
4. Standard dilution was made on the microwell plate as follows (Figure 19):

   - 100 µl of Sample Diluent were added in duplicate to all standard wells.
   - 100 µl of prepared standard were pipetted (concentration = 64 ng/ml) in duplicate into well A1 and A2. The contents of wells A1 and A2 were mixed by repeated aspiration and ejection (concentration of standard 1, S1 = 32 ng/ml), and 100 µl were transferred to wells B1 and B2, respectively.
   - This procedure was repeated 5 times, creating two rows of human Bcl-2 standard dilutions ranging from 32 to 0.5 ng/ml. 100 µl of the contents from the last microwells (G1, G2) used were discarded.

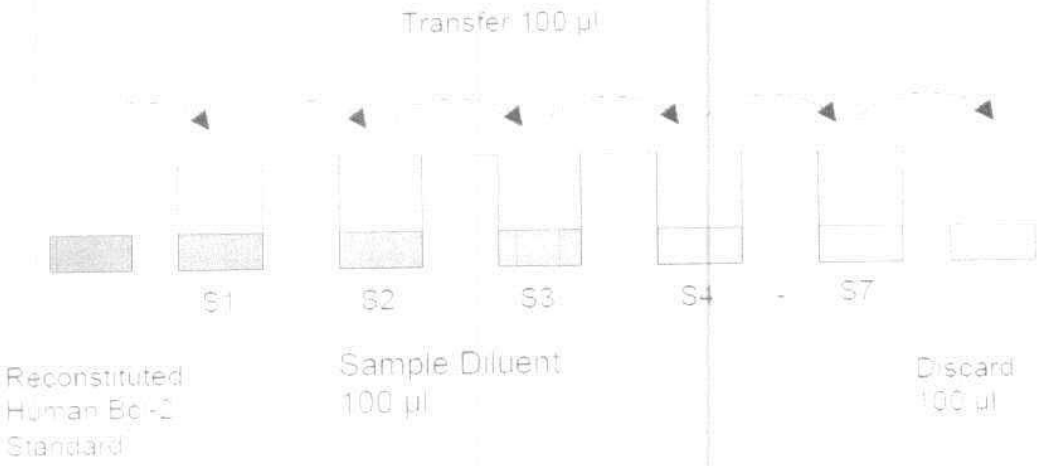

Figure 19: Steps of preparation of standard dilutions of Bcl-2.

5. 100 µl of Sample Diluent were added in duplicate to the blank wells.
6. 80 µl of Sample Diluent were added to the sample wells.
7. 20 µl of each sample were added in duplicate to the sample wells.
8. 50 µl of Biotin-Conjugate were added to all wells.
9. Plate was covered with an adhesive film and incubated at room temperature (18 to 25°C) for 2 hour, if available on a microplate shaker set at 100 rpm.
10. Adhesive film was removed and wells were emptied. Microwell strips were washed 3 times.
11. 100 µl of diluted Streptavidin-HRP were added to all wells, including the blank wells.
12. Plate was covered with an adhesive film and incubated at room temperature (18 to 25°C) for 1 hour.

13. Adhesive film was removed and wells were emptied. Microwell strips were washed 3 times.
14. 100 µl of TMB Substrate Solution were added to all wells.
15. Microwell strips were incubated at room temperature (18° to 25°C) for about 10 min.
16. The color development on the plate was monitored and the substrate reaction stopped before positive wells are no longer properly recordable. The stop solution was added when the highest standard has developed a dark blue color.
17. The enzyme reaction was stopped by quickly and uniformly pipetting 100 µl of Stop Solution into each well. Results were read immediately after the Stop Solution was added.
18. Absorbance of each microwell was read on a spectrophotometer using 450 nm.

Calculation of Results

1. The average absorbance values were calculated for each set of duplicate standards and samples.
2. A standard curve was created by plotting the mean absorbance for each standard concentration on the Y-axis against the human Bcl-2 concentration on the X-axis of log-log paper. A best fit curve was drawn through the points of the graph. (Figure 20) represents the standard curve of human Bcl-2.
3. The concentration of circulating human Bcl-2 for each sample was determined from the standard curve.
4. As samples were diluted 1:5 (20 µl sample + 80 µl Sample Diluent), the concentration read from the standard curve was multiplied by the dilution factor (x 5).

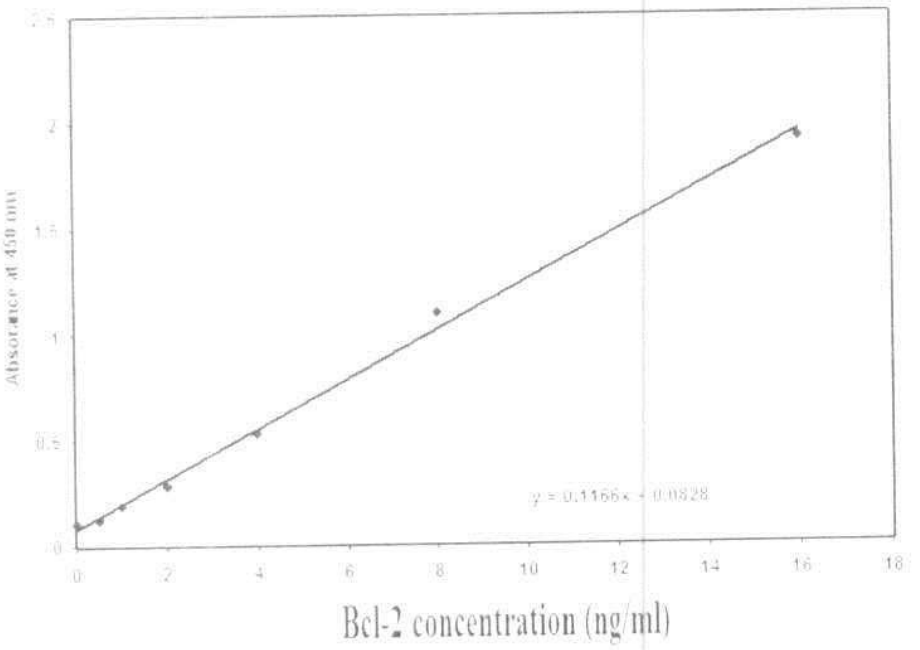

Figure 20: Standard curve for human Bcl-2.

Determination of serum Bax levels

Bax was measured by commercially available ELISA kit for the quantitative detection of human Bax. USCN, USA.[49]

Principle

The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to Bax. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for Bax. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain Bax, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibited a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm. The concentration of Bax in the samples is then determined by comparing the O.D of the sample to the standard curve.

Reagents Provided

- 1 pre-coated ready to use 96 well strip plate
- 4 Plate sealer for 96 wells
- 2 vial standard
- 1 vial Standard diluents
- 1 vial Assay diluents A
- 1 vial Assay diluents B
- 1 vial TMB substrate
- 1 vial Wash buffer
- 1 vial Stop solution

Preparation of Reagents

1. All kit components and samples were brought to room temperature.

2. Standard:-

   The standard was reconstituted with 1.0 ml of standard diluents, kept for 10 minutes at room temperature and shacked gently. The concentrations of the standard solution were 0, 25, 50, 75, 100, 125, 150 and 200 ng/ml, preparation of these standard solutions was done using the dilution law, considering the 200 ng/ml as the stock solution.

3. Assay diluents A and Assay diluents B:-

   6 ml of diluents A or B concentrate (2x) was diluted with 6 ml of distilled water to prepared 12 ml of assay diluent A or B.

4. Detection reagent A and Detection B:-

   The working concentration was diluted with working assay diluents A or B, respectively (1:100).

5. Wash solution:-

   20 ml of wash solution concentrated (30x) was diluted with 580 ml of distal water to prepared 600 ml of wash solution (1x).

Assay procedure

All reagents were reached at room temperature before use. All liquid reagents prior to use were gently mixed.

1. 100µl. of each standard, blank and samples were added into the appropriate wells. The microwells plate was covered with the plate sealer and incubated for 2 hours at 37°C.
2. The liquid was removed from each well without wash.
3. 100µl. of Detection Reagent A working solution were added to each well and incubated for 1 hour at 37°C after covering it with the plate sealer.
4. The solution was aspirated and each well was washed with 350µl. of diluted Wash Solution and was allowed to sit for 1-2 minutes. The remaining liquid was removed from all wells completely by snapping the plate onto absorbent paper. This wash was repeated 3 times. After the last wash, any remaining Wash Buffer was removed by aspiration or decantation. The plate was inverted against absorbent paper.
5. 100µl. of Detection Reagent B working solution was added to each well then incubated for 30 minutes at 37°C after covering it with the plate sealer.
6. The aspiration/wash process was repeated for five times as conducted in step 4.
7. 90µl. of Substrate Solution was added to each well then covered with a new Plate sealer and incubate for 15 - 25 minutes at 37°C (Don't exceed 30 minutes) in the dark. The liquid was turned blue by addition of substrate solution.

8. 50µl of Stop Solution was added to each well. The liquid was turned yellow by the addition of stop solution.
9. The color intensity of each microwell was measured at 450 nm using an ELISA reader.

Calculation of Results

1. The average of absorbance values were calculated for each set of duplicate standards and samples.
2. A standard curve was created by plotting the mean absorbance for each standard concentration on the Y-axis against the human Bax concentration on the X-axis of log-log paper. A best fit curve was drawn through the points of the graph. (Figure 21) represents the standard curve of human Bax.
3. The concentration of circulating human Bax for each sample was determined from the standard curve.

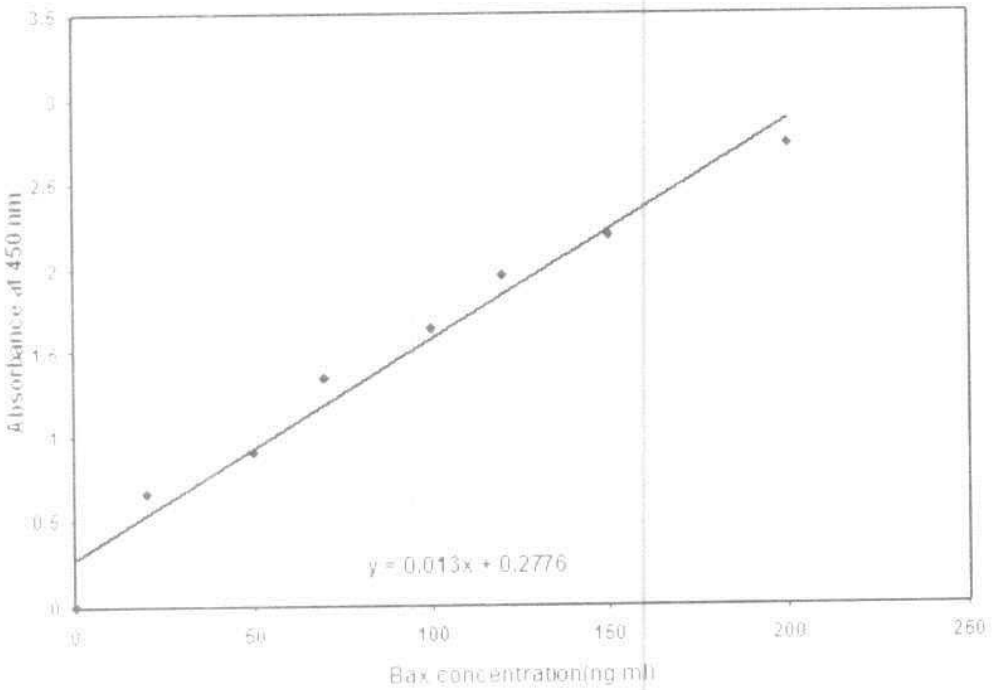

Figure 21:- Standard curve for human Bax

Determination of serum p53 level

P53 was measured by commercially available ELISA kit for the quantitative detection of human p53. Bender MedSystems GmbH Campus Vienna, Austria, Europe.

Principles of the Assay:

1. An anti-human P53 coating antibody is adsorbed onto microwells (Figure 22a).

Figure 22a

2. Human P53 present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human P53 antibody is added and binds to human P53 captured by the first antibody (Figure 22b).

Figure 22b

3. Following incubation unbound biotin-conjugated anti-human P53 antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human P53 antibody (Figure 22c).

Figure 22c

4. Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells (Figure 22d).

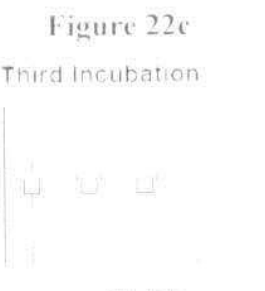

Figure 22d

5. A colored product is formed in proportion to the amount of human P53 present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human P53 standard dilutions and human P53 sample concentration determined (Figure 22e).

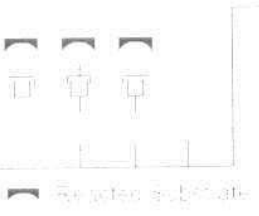

Figure 22e

Reagents Provided

- 1 aluminium pouch with a Microwell Plate coated with monoclonal antibody to human sFasL.
- 1 vial (100 µl) Biotin-Conjugate anti-human sFasL monoclonal antibody
- 1 vial (150 µl) Streptavidin-HRP
- 1 vials human P53 Standard lyophilized. 100 U/ml upon reconstitution
- 1 vial (12 ml) Sample Diluent
- 1 vial (5 ml) Assay Buffer Concentrate 20x
  (PBS with 1% Tween 20 and 10% BSA)
- 1 bottle (50 ml) Wash Buffer Concentrate 20x
  (PBS with 1% Tween 20)
- 1 vial (15 ml) Substrate Solution (tetramethyl-benzidine)
- 1 vial (15 ml) Stop Solution (1M Phosphoric acid)
- 4 Adhesive Films

Preparation of Reagents

Buffer Concentrates were brought to room temperature and were diluted before starting the test procedure.

1. 50 ml of the Wash Buffer Concentrate (20x) was poured into a clean 1000 ml graduate cylinder and brought to a final volume of 1000 ml with distilled water.
2. 5 ml of the Assay Buffer Concentrate (20x) were poured into a clean 100 ml graduate cylinder and brought to a final volume of 100 ml with distilled water.
3. 1:100 dilution of the concentrated Biotin-Conjugate prepared by 60 µl of Biotin-Conjugate with 5.94 ml of (1x) assay buffer in a clean plastic tube.
4. 1:100 dilution of the concentrated Streptavidin-HRP solution by 120 µl of Streptavidin-HRP solution with 11.880 ml of (1x) assay buffer in a clean plastic tube.
5. Human P53 Standard was reconstituted by addition of distilled water then mixed gently to insure complete and homogeneous solubilization. (Concentration of reconstituted standard 100 U/ml). Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

Test Protocol

1. Each sample, standard, blank and optional control samples should be assayed in duplicate.
2. Microwells strips were washed twice with approximately 400 µl Wash Buffer per well with thorough aspiration of microwell contents between washes. Wash Buffer was allowed to sit in the wells for about 10 – 15 seconds before aspiration.
3. After the last wash step, wells were emptied and microwell strips were tapped on absorbent paper to remove excess Wash Buffer.
4. Standard dilution was made on the microwell plate as follows (Figure 23):
   - 100 µl of Sample Diluent were added in duplicate to all standard wells.
   - 100 µl of prepared standard were pipetted (concentration = 100 U/ml) in duplicate into well A1 and A2. The contents of wells A1 and A2 were mixed by repeated aspiration and ejection (concentration of standard 1, S1 = 50 U/ml), and 100 µl were transferred to wells B1 and B2, respectively.
   - This procedure was repeated 5 times; creating two rows of human P53 standard dilutions ranging from 50 to 0.78 U/ml. 100 µl of the contents from the last microwells (G1, G2) used were discarded.

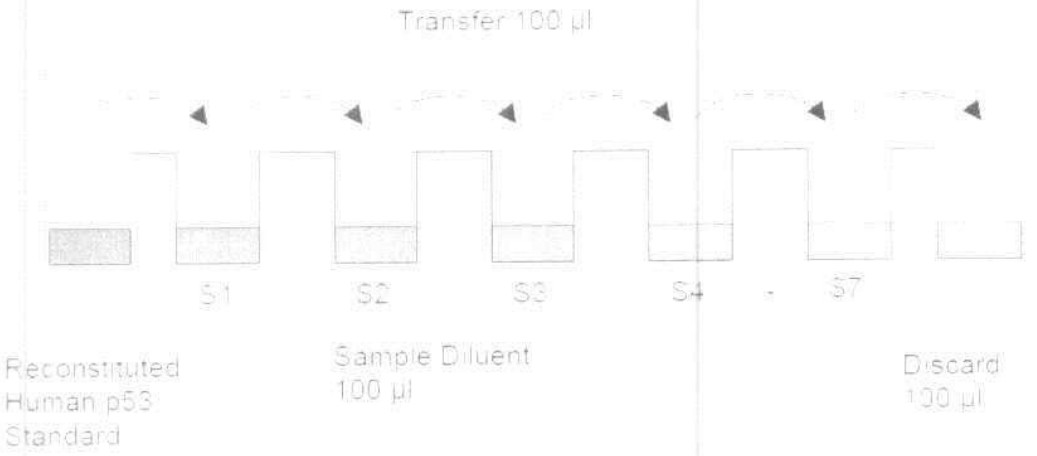

Figure 23: Steps of preparation of standard dilutions of P53.

5. 100 µl of Sample Diluent were added in duplicate to the blank wells.
6. 50 µl of Sample Diluent were added to the sample wells.
7. 50 µl of each sample were added in duplicate to the sample wells.
8. 50 µl of Biotin-Conjugate were added to all wells.
9. Plate was covered with an adhesive film and incubated at room temperature (18 to 25° C) for 2 hours on a microplate shaker set at 100 rpm.
10. Adhesive film was removed and wells were emptied. Microwell strips were washed 3 times.
11. 100 µl of diluted Streptavidin-HRP were added to all wells, including the blank wells.
12. Plate was covered with an adhesive film and incubated at room temperature (18° to 25° C) for 1 hour on a microplate shaker set at 100 rpm.
13. Adhesive film was removed and wells were emptied. Microwell strips were washed 3 times.
14. 100 µl of TMB Substrate Solution were pipetted to all wells.

15. Microwell strips were incubated at room temperature (18° to 25° C) for about 10 min.
16. The color development on the plate was monitored and the substrate reaction stopped before positive wells are no longer properly recordable. The stop solution was added when the highest standard has developed a dark blue color.
17. The enzyme reaction was stopped by quickly and uniformly pipetting 100 µl of Stop Solution into each well. Results were read immediately after the Stop Solution is added.
18. Absorbance of each microwell was read on a spectrophotometer using 450 nm.

Calculation of Results

1. The average absorbance values were calculated for each set of duplicate standards and samples.
2. A standard curve was created by plotting the mean absorbance for each standard concentration on the Y-axis against the human P53 concentration on the X-axis log-log paper. A best fit curve was drawn through the points of the graph. (Figure 24) represents the standard curve of human P53.
3. The concentration of circulating human P53 was determined form the standard curve.
4. As samples were diluted 1:2 (50 µl sample + 50 µl Sample Diluent), the concentration read from the standard curve was multiplied by the dilution factor (x 2).

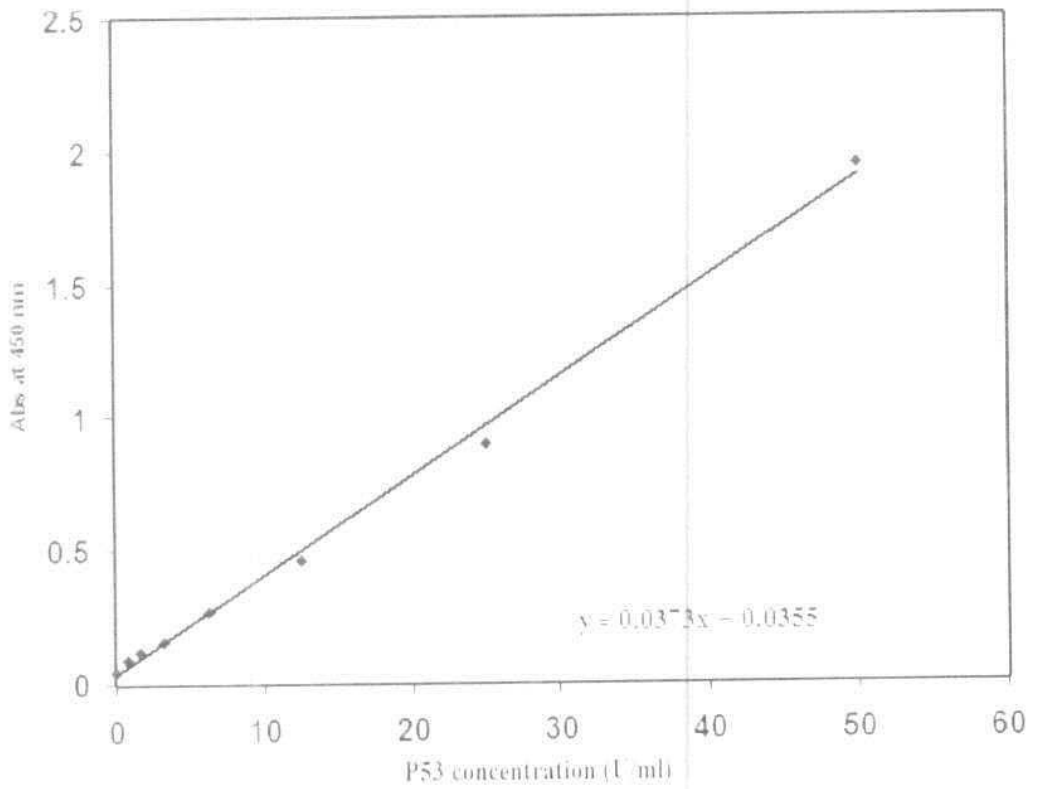

Figure 24: Standard curve for human P53.

Determination of serum CA15.3 levels

Principle

CA 15-3-IRMA is a two-step Immunoradiometric assay based on coated-tube separation. Mab1, the capture antibody, is attached to the lower and inner surface of the plastic tube. Add calibrators or samples to the tubes. After incubation, washing removes the occasional excess of antigen. Addition of Mab2, the signal antibody labeled with ^{125}I, will complete the system and trigger the immunological reaction. After washing, the remaining radioactivity bound to the tube reflects the antigen concentration.[137]

Reagents

- Anti-CA 15.3 monoclonal antibodies coated tubes.
- Anti-CA 15.3 (monoclonal antibody) in phosphate buffer with bovine serum and sodium azide (<0.1%).
- Calibrators 1-5 in phosphate buffer with bovine serum albumin and thymol (<0.1%).
- Dilution buffer: phosphate buffer with bovine serum and sodium azide (<0.1%).
- Wash buffer concentration: 70 x (TRIS-HCl).
- Controls - N1 or 2 in human serum and thymol.

N.B. The dilution buffer was used as zero calibrator.

Reagent preparation

- Calibrators: The calibrators were reconstituted with 0.5 ml dilution buffer.
- Controls: The controls were reconstituted with 0.5 ml distilled water.
- Working Wash solution: An adequate volume of Working Wash was prepared by adding 69 volumes of distilled water to 1 volume of Wash Solution (70x). A magnetic stirrer was used to homogenize.

Procedure

1. 500 µl of dilution buffer was dispensed into each tube.
2. 20 µl of each sample and control was added into these tubes.
3. Calibrators, pre-diluted samples and controls were briefly vortexed and 50 µl of each were dispensed into the respective tubes.
4. Incubation for 90 minutes at room temperature was done on a tube shaker (400 rpm).
5. The content of each tube was decanted. (Except total counts)

6. The tubes were washed with 2 ml working wash solution (except total counts) and were aspirated. Foaming during the addition of the working wash solution was avoided.

7. The tubes were washed again with 2 ml wash solution (except total counts) and were decanted.

8. The tubes were let stand upright for 2 minutes and the remaining drops of the liquid were decanted.

9. 50 µl of ^{125}I labeled anti CA15.3 was dispensed into each tube, including the uncoated tubes for the total counts.

10. Incubation for 90 minutes at room temperature was done on a tube shaker (400 rpm).

11. The contents of each tube (except total counts) were decanted.

12. The tubes were washed with 2 ml working wash solution (except total counts) and were decanted. Foaming during the addition of the working wash solution was avoided.

13. The tubes were washed again with 2 ml wash solution (except total counts) and were decanted.

14. The tubes were let stand upright for 2 minutes and the remaining drops of the liquid were decanted.

15. The tubes were counted in a gamma counter for 60 seconds.

Calculation of results

Computer assisted data reduction was applied to calculate unknown serum CA15.3 levels by counting for 1 minute in a gamma counter (Perkin-Elmer, Finland). A 5-parameter logistic function curve was used to determine the unknown CA15.3 serum levels.

Statistical Analysis:

Statistical analyses were performed using SPSS 11.5 software package. Quantitative data were described using minimum and maximum as well as mean and standard error.

The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test, D'Agstino test was used if there was a conflict between the two previous tests. The data were abnormally distributed so, non-parametric tests were used.

The Non-parametric Mann–Whitney U-test was used for studying differences between breast cancer patients group and control group regarding serum Bcl-2, Bax, p53 and CA15.3 for relating studied parameters concentrations to tumor size, lymph node status, Her-2, vascular invasion, stage, grade, PR and ER. The non-parametric Kruskal-Wallis test was used to study the differences in serum parameters before and after surgery and after chemotherapy. The Non-parametric Spearman's test was used to investigate correlations between different serum parameters.

The diagnostic values of serum Bcl-2, P53 and CA15.3 were compared using the Receiver Operating Characteristic (ROC) curve analysis. To obtain the optimal cut off point for each parameter in predicting breast cancer, we chose the point on the ROC curve which represented the largest sum of sensitivity and specificity, [175] or equivalently, of the Youden index: sensitivity specificity. The area under the ROC curve (AUROC) was used as a general measure of discrimination of a predictor. To assess whether the difference in the areas under two ROC curves is of statistical significance we used the procedure described in Basic principles of ROC analysis. [176] Area more than 50% gives acceptable performance and area about 100% is the best performance for the test.

Univariate survival analysis of the studied parameters was assessed using the Kaplan Meier method and log rank test was used for survival time. Statistical differences between survival curves were evaluated using the log-rank test. *P* values less than 0.05 were accepted as significant.

RESULTS

RESULTS

Clinicopathological parameters characterizing breast cancer patients group:

The general characterizations of breast cancer patients group were represented in table (3). The table showed that all cases were of histological grades II (83%) and grade III (17%) and clinical stage II (70%) and stage III (30%). With respect to tumor size (60 %) of cases had tumor size (< 5 cm) while 40 % were (>5 cm). lymph node involvement were pathologically detected in 37% of cases while 63% were free. 40% of cases were negative estrogen receptor (ER), while 60% were positive. 47% of cases were negative progesterone receptor (PR), while 53% were positive. 57% of cases were negative Her-2 while 43% were positive. Vascular invasion was observed in 70% of cases, while 30% were positive. Furthermore 2 patients only (6.7%) developed metastasis during follow up period (2 years).

Table (3): Clinicopathological parameters and general characterization of patients of malignant group involving histological grade, clinical stage, tumor size, lymph node involvement, ER, PR status, Her-2 and vascular invasion.

Parameter		Number (N)	Percent (%)
Histological Grade	II	25	83
	III	5	17
Clinical stage	II	21	70
	III	9	30
Tumor size	< 5	18	60
	≥ 5	12	40
Lymph node Involvement	-ve	19	63
	+ve	11	37
ER status	-ve	12	40
	+ve	18	60
PR status	-ve	14	47
	+ve	16	53
Her-2	-ve	17	57
	+ve	13	43
Vascular Invasion	-ve	9	30
	+ve	21	70
Metastasis	-ve	28	93.3
	+ve	2	6.7

Biochemical results

Results of serum Bcl-2 (ng/ml)

Individual data range and mean ± S.E. values of Bcl-2 (ng/ml) in normal control subjects, and breast cancer patients group, before surgery, one month after surgery and after 6 cycles of chemotherapy, were shown in table (4) and illustrated in (Figure 25). Statistical analyses of these results were represented in table (5).

As presented in table (4), serum Bcl-2 concentration (ng/ml) was ranged from 0.000 to 3.096 with a mean value of 0.937 ± 0.166 in normal control subjects, from 0.146 to 7.368 with a mean value of 2.854 ± 0.327 in breast cancer patients before surgery, from 0.866 to 4.737 with a mean value of 2.35 ± 0.206 one month after surgery and from 0.00 to 6.612 with a mean value of 1.943 ± 0.245 after six cycles of chemotherapy.

The statistical analyses of these results revealed that the levels of serum Bcl-2 of breast cancer patients either before or after one month of surgery as well as after 6 cycle of chemotherapy were significantly higher than those of control group. It was also noticed that serum Bcl-2 level of breast cancer patients which was significantly similar to those after one month of surgery, significantly decreased after 6 cycles of chemotherapy. On comparing the level of this parameter after 6 cycles of chemotherapy with those after one month of surgery the difference was significant.

Table (4) Serum Bcl-2 values (ng/ml) in normal control subjects and breast cancer patients group before surgery, one month after surgery and after 6 cycles of chemotherapy

No	Normal Control Subjects (n = 30)	Breast cancer patients group (n = 30)		
		Before surgery	After 1 month of surgery	After 6 cycles of chemotherapy
1	0.629	2.324	1.566	1.166
2	1.807	2.690	2.566	1.823
3	0.000	2.515	1.166	0.995
4	0.526	5.515	3.353	1.352
5	2.110	4.698	4.395	2.995
6	2.924	3.087	3.780	1.543
7	1.423	0.659	1.823	1.166
8	2.410	0.146	3.780	3.754
9	0.738	7.368	3.096	6.612
10	0.180	2.589	1.954	1.258
11	0.000	2.251	1.360	2.048
12	0.008	1.586	1.265	0.851
13	3.096	1.300	1.995	0.738
14	1.396	2.660	0.866	2.324
15	0.480	3.189	1.724	2.153
16	0.000	0.780	1.566	0.652
17	0.523	5.172	3.995	4.125
18	0.609	4.125	0.866	1.895
19	0.738	0.652	3.439	3.139
20	0.700	0.509	2.024	1.252
21	2.410	2.659	1.934	1.738
22	0.394	2.659	1.137	1.609
23	1.756	5.146	1.509	0.000
24	0.0943	2.024	2.252	1.537
25	0.1372	4.859	4.738	0.995
26	0.992	1.510	3.595	2.067
27	0.892	0.943	1.515	0.309
28	0.620	2.660	1.124	2.067
29	0.237	5.407	2.866	3.988
30	0.278	3.940	3.238	2.153
Range	0.000-3.096	0.146 - 7.368	0.866 - 4.737	0.000 - 6.612
Mean ± SE	0.937 ± 0.166	2.854 ± 0.327	2.350 ± 0.206	1.943 ± 0.245

Table (5): The statistical analysis of serum Bcl-2 values (ng/ml) in normal control subjects and breast cancer patients group before surgery, one month after surgery and after 6 cycles of chemotherapy

	Normal Control Subjects (n = 30)	Breast cancer patients group (n = 30)		
		Before surgery	After one month of surgery	After 6 cycles of chemotherapy
Serum Bcl-2	0.937 ± 0.16	2.854 ± 0.327	2.35 ± 0.206	1.943 ± 0.245
p_1		0.001*	0.001*	0.001*
p_2			0.09	0.01#
p_3				0.049

P1: values compared to normal control subjects.
P2: values compared to breast cancer patients group before surgery.
P3: values compared to breast cancer patients group after one month of surgery.
* : Significantly different from control group.
# : Significantly different from breast cancer patients group before surgery.
@ : Significantly different from breast cancer patients group after one month of surgery.
Significance was considered at the level of P- value <0.05.

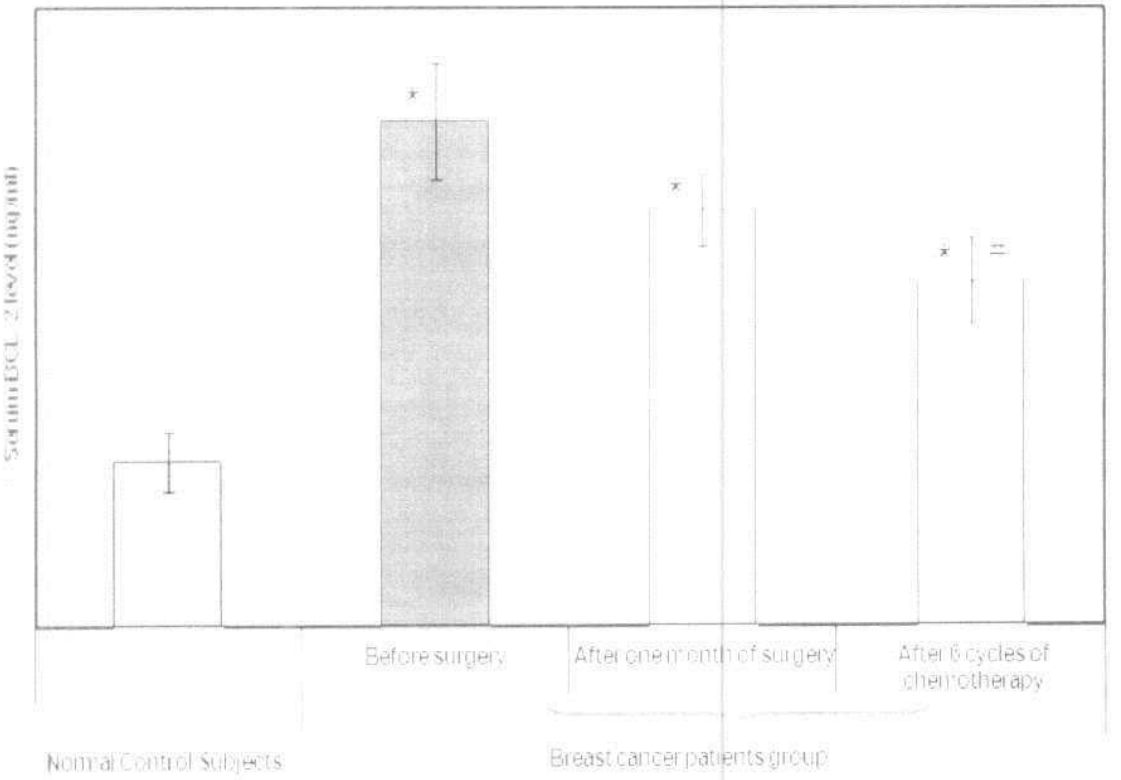

Figure 25: Bar chart representing the Mean ± S.E of Bcl-2 (ng/ml) in normal control subjects and breast cancer patients before surgery, after one month of surgery and after 6 cycles of chemotherapy.

* : Significantly different from control group.
\# : Significantly different from breast cancer patients group before surgery.
: Significantly different from breast cancer patients group after one month of surgery.
Significance was considered at the level of P- value <0.05.

Results of serum Bax (ng/ml)

Individual data range and mean ± S.E values of Bax (ng/ml) in normal control subjects, and breast cancer patients group, before surgery, one month after surgery and after 6 cycles of chemotherapy, were shown in table (6) and illustrated in (Figure 26). Statistical analyses of these results were represented in table (7).

As presented in table (6), serum Bax concentration (ng/ml) was ranged from 30.72 to 108.7 with a mean value of 75.7 ± 3.94 in normal control subjects, from 10.49 to 97.14 with a mean value of 67.1 ± 3.6 in breast cancer patients before surgery, from 10.8 to 101.72 with a mean value of 68.5 ± 3.74 one month after surgery and from 12.88 to 129.51 with a mean value of 65.37 ± 5.21 after 6 cycles of chemotherapy.

The statistical analyses of these results revealed that the level of serum Bax in breast cancer patients either before or after one month of surgery as well as after 6 cycles of chemotherapy were nearly within the same range and insignificantly less than those of control group.

Table (6): Serum Bax values (ng/ml) in normal control subjects and breast cancer patients group before surgery, one month after surgery and after 6 cycles of chemotherapy

No	Normal Control Subjects (n = 30)	Breast cancer patients group (n = 30)		
		Before surgery	After 1 month of surgery	After 6 cycles of chemotherapy
1	80.22	95.50	101.72	106.80
2	99.89	43.49	47.03	65.65
3	102.86	83.34	83.95	51.11
4	39.82	50.88	55.97	92.07
5	108.70	64.49	62.81	46.49
6	66.02	77.65	72.95	62.41
7	80.79	40.36	31.26	20.40
8	90.17	92.91	50.26	46.11
9	79.09	85.11	79.72	56.65
10	59.19	64.88	63.34	91.57
11	57.67	90.25	48.36	97.28
12	55.32	65.88	68.49	83.88
13	80.20	62.11	69.27	68.03
14	80.20	58.24	52.68	66.21
15	53.72	71.42	71.95	25.26
16	42.49	64.13	60.94	66.63
17	107.49	97.14	91.03	104.26
18	30.72	82.34	88.27	55.19
19	70.22	78.21	75.03	58.99
20	100.89	43.37	67.42	25.55
21	102.86	64.27	68.15	41.55
22	69.82	75.94	77.18	65.25
23	108.70	10.49	97.21	129.51
24	66.02	52.36	67.81	62.56
25	60.79	42.11	97.34	74.65
26	60.17	60.71	45.88	36.40
27	82.09	54.65	10.80	12.88
28	64.19	70.11	71.03	74.11
29	72.67	78.58	76.82	56.32
30	98.37	92.05	100.21	117.25
Range	30.72 - 108.70	10.49-97.14	10.80 - 101.72	12.88 – 129.51
Mean ± SE	75.7 ± 3.94	67.1 ± 3.6	68.5 ± 3.74	65.37 ± 5.21

Table (7): The statistical analysis of serum Bax values (ng/ml) in normal control subjects and breast cancer patients group before surgery, one month after surgery and after 6 cycles of chemotherapy

	Normal Control Subjects (n=30)	Breast cancer patients group (n=30)		
		Before surgery	After 1 month of surgery	After 6 cycles of chemotherapy
Bax	75.7 ± 3.94	67.1 ± 3.6	68.5 ± 3.74	65.37 ± 5.21
p_1		0.147	0.231	0.095
p_2			0.877	0.360
p_3				0.441

P1: values compared to normal control subjects.
P2: values compared to breast cancer patients group before surgery.
P3: values compared to breast cancer patients group after one month of surgery.

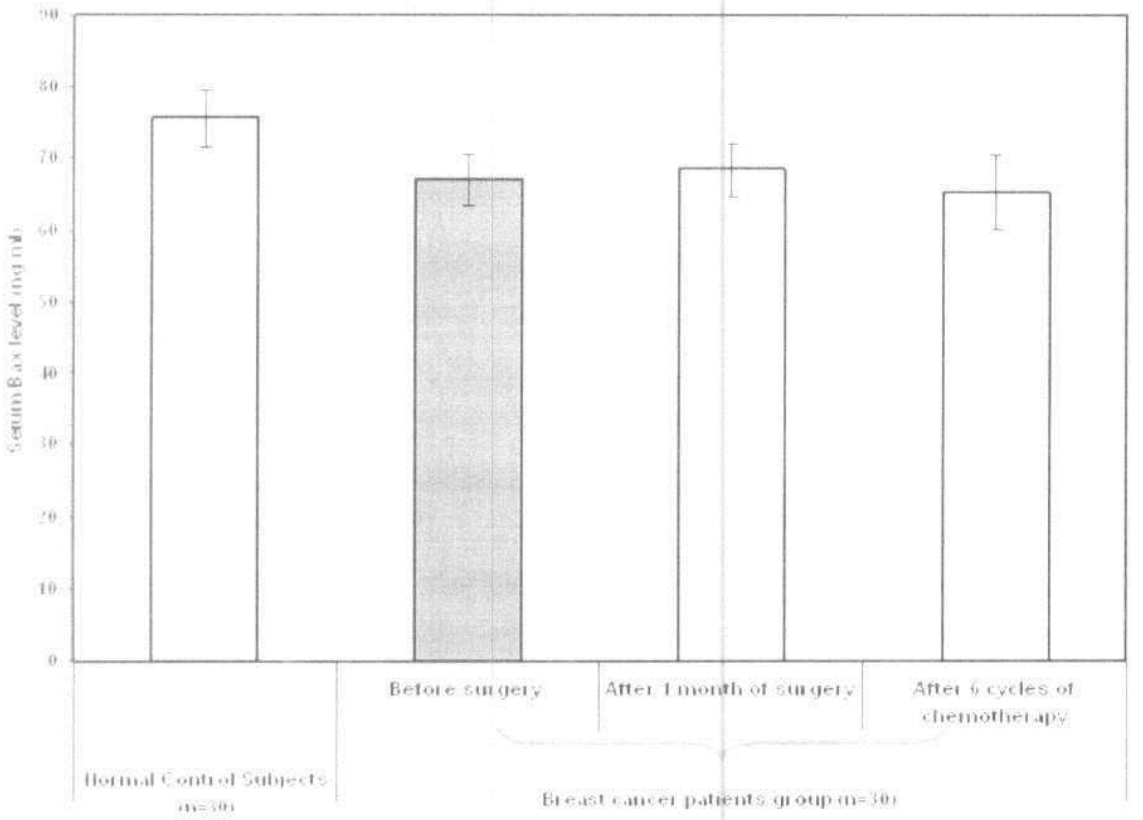

Figure 26: Bar chart representing the Mean ± S.E of Bax (ng/ml) in normal control subjects and breast cancer patients before surgery, after one month of surgery and after 6 cycles of chemotherapy.

Results of serum P53 (U/ml)

Individual data, range and mean ± S.E values of P53 (U/ml) in normal control subjects, and breast cancer patients group, before surgery, one month after surgery and after 6 cycles of chemotherapy, were shown in table (8) and illustrated in (Figure 27). Statistical analyses of these results were represented in table (9).

As presented in table (8), serum P53 concentration (U/ml) was ranged from 2.177 to 5.188 with a mean value of 3.327 ± 0.133 in normal control subjects, from 1.58 to 4.059 with a mean value of 2.411 ± 0.104 in breast cancer patients before surgery, from 1.159 to 5.134 with a mean value of 2.305 ± 0.143 one month after surgery and from 1.030 to 4.759 with a mean value of 2.255 ± 0.153 after six cycles of chemotherapy.

The statistical analyses of these results revealed that the levels of serum P53 of breast cancer patients either before or after one month of surgery as well as after 6 cycle of chemotherapy were significantly less than those of control group. It was also noticed that serum P53 level of breast cancer patients before surgery was significantly decreased after 6 cycle of chemotherapy. On comparing the level of this parameter after one month of surgery and those after 6 cycle of chemotherapy the difference was not significant.

Table (8): Serum P53 values (U/ml) in normal control subjects and breast cancer patients group before surgery, one month after surgery and after 6 cycles of chemotherapy

No	Normal Control Subjects (n = 30)	Breast cancer patients group (n = 30)		
		Before surgery	After 1 month of surgery	After 6 cycles of chemotherapy
1	3.800	2.930	3.145	2.823
2	2.394	3.306	2.715	2.823
3	2.823	2.715	2.661	2.446
4	3.091	1.640	1.694	1.694
5	2.177	1.855	2.016	1.801
6	2.285	2.823	2.876	2.661
7	3.145	2.124	1.962	1.747
8	2.715	2.661	2.339	2.392
9	3.199	1.962	2.554	2.554
10	3.952	2.553	1.963	1.549
11	5.188	1.580	1.159	1.368
12	2.984	2.661	2.930	2.876
13	2.930	2.339	2.124	2.339
14	3.629	2.070	2.016	1.855
15	3.038	2.070	2.339	1.855
16	3.844	2.124	1.532	1.855
17	3.360	3.597	5.134	4.758
18	3.360	1.962	1.694	1.747
19	3.683	4.059	3.414	4.059
20	3.522	2.339	1.909	1.747
21	3.737	2.345	1.325	1.031
22	3.360	2.608	2.070	2.231
23	3.280	2.124	2.177	2.339
24	4.800	2.694	2.145	2.780
25	4.860	1.664	1.456	1.280
26	2.439	2.715	3.145	3.306
27	3.234	2.446	2.446	2.070
28	3.146	2.608	2.876	3.145
29	3.489	1.824	1.576	1.259
30	2.349	1.924	1.772	1.269
Range	2.177 – 5.188	1.58 – 4.059	1.159 – 5.134	1.030 – 4.759
Mean ± SE	3.327 ± 0.133	2.411 ± 0.104	2.305 ± 0.143	2.255 ± 0.153

Table (9): The statistical analysis of serum P53 values (U/ml) in normal control subjects and breast cancer patients group before surgery, one month after surgery and after 6 cycles of chemotherapy

	Normal Control Subjects (n = 30)	Breast cancer patients group (n = 30)		
		Before surgery	After 1 month of surgery	After 6 cycles of chemotherapy
Serum P53	3.327 ± 0.133	2.4106 ± 0.1044	2.305 ± 0.1435	2.255 ± 0.153
P_1		< 0.001*	< 0.001*	< 0.001*
P_2			0.122	0.049#
P_3				0.198

P1: values compared to normal control subjects.
P2: values compared to breast cancer patients group before surgery.
P3: values compared to breast cancer patients group after one month of surgery.
* : Significantly different from control group.
# : Significantly different from breast cancer patients group before surgery.
Significance was considered at the level of P- value <0.05.

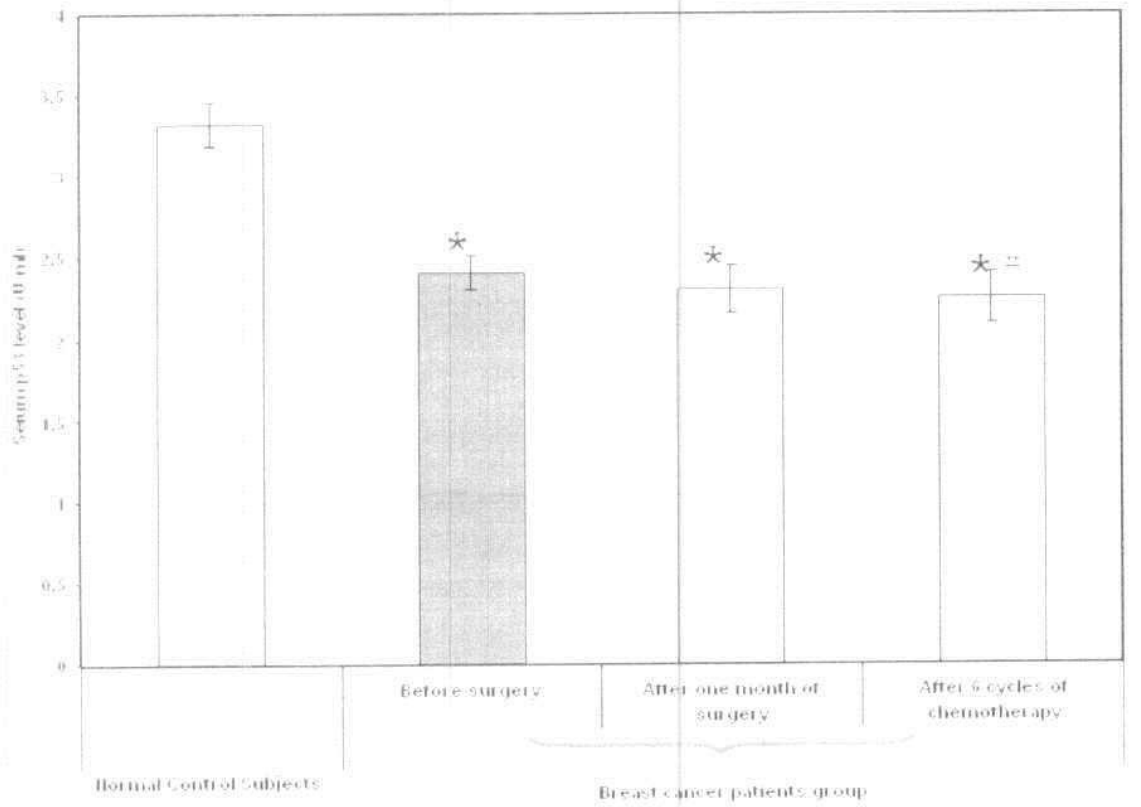

Figure (27): Bar chart representing the Mean ± S.E of P53 (U/ml) in normal control subjects and breast cancer patients before surgery, after one month of surgery and after 6 cycles of chemotherapy.

* : Significantly different from control group.
\# : Significantly different from breast cancer patients group before surgery.
Significance was considered at the level of P- value <0.05.

Results of serum CA 15.3 (IU/ml)

Individual data range and mean ± S.E values of CA 15.3 (IU/ml) in normal control subjects, and breast cancer patients group, before surgery, one month after surgery and after 6 cycles of chemotherapy, were seen in table (10) and illustrated in (Figure 28). Statistical analyses of these results are represented in table (11).

As presented in table (10), the serum CA15.3 concentration (IU/ml) was ranged from 9.145 to 29.267 with a mean value of 16.94 ± 0.835 in normal control subjects, from 10.447 to 56.149 with a mean value of 24.92 ± 1.823 in breast cancer patients before surgery, from 11.01 to 55.368 with a mean value of 23.945 ± 1.77 one month after surgery and from 10.015 to 65.772 with a mean value of 20.61 ± 1.89 after six cycles of chemotherapy.

The statistical analyses of these results revealed that the levels of serum CA15.3 in breast cancer patients either before or after one month of surgery were nearly within the same range and significantly higher than those of control group. After 6 cycle of chemotherapy, the level of the parameter was insignificantly higher than those of control group, significantly decrease than those of before surgery and showed insignificant difference when compared with those after one month of surgery.

Table (10) Serum CA15.3 values (IU/ml) in normal control subjects and breast cancer patients group before surgery, one month after surgery and after 6 cycles of chemotherapy

No	Normal Control Subjects (n =30)	Breast cancer patients group (n = 30)		
		Before surgery	After 1 month of surgery	After 6 cycles of chemotherapy
1	17.000	35.490	12.671	12.816
2	29.267	12.347	14.262	14.331
3	13.424	39.070	11.010	11.795
4	17.245	24.322	24.972	22.277
5	18.405	27.000	29.359	20.888
6	12.799	10.447	36.822	34.200
7	22.256	16.300	55.368	19.563
8	16.371	23.415	20.368	24.682
9	17.632	18.788	19.589	11.598
10	15.128	28.356	26.358	22.037
11	20.494	22.359	16.289	11.369
12	10.371	27.500	22.037	19.236
13	17.808	18.199	17.047	20.008
14	18.409	13.658	19.607	19.074
15	12.729	11.847	26.741	24.020
16	18.040	18.612	17.620	18.372
17	19.813	22.398	17.654	23.489
18	17.857	34.348	38.390	65.772
19	17.472	16.028	17.794	22.589
20	22.584	19.740	19.740	12.149
21	10.629	25.900	11.254	11.964
22	19.251	37.994	37.397	28.258
23	13.500	28.898	25.954	20.751
24	13.500	12.250	32.876	17.125
25	10.500	29.486	29.025	19.241
26	23.247	33.296	27.549	27.839
27	9.145	56.149	14.255	19.067
28	17.365	24.615	19.408	22.669
29	12.589	25.479	25.476	10.015
30	23.421	33.280	31.458	11.046
Range	9.145 29.267	10.447 56.149	11.01 55.368	10.015 65.772
Mean ± SE	16.94 ± 0.835	24.92 ± 1.823	23.945 ± 1.77	20.61 ± 1.89

Table (11): The statistical analysis of serum CA15.3 values (IU/ml) in normal control subjects and breast cancer patients group before surgery, one month after surgery and after 6 cycles of chemotherapy.

	Normal Control Subjects (n = 30)	Breast cancer patients group (n = 30)		
		Before surgery	After one month of surgery	After 6 cycles of chemotherapy
Serum CA15.3	16.94 ± 0.835	24.92 ± 1.823	23.945 ± 1.77	20.61 ± 1.89
p_1		0.001*	0.002*	0.076
p_2			0.349	0.043#
p_3				0.072

P1: values compared to normal control subjects.
P2: values compared to breast cancer patients group before surgery.
P3: values compared to breast cancer patients group after one month of surgery.
* : Significantly different from control group.
# : Significantly different from breast cancer patients group before surgery.
Significance was considered at the level of P- value <0.05.

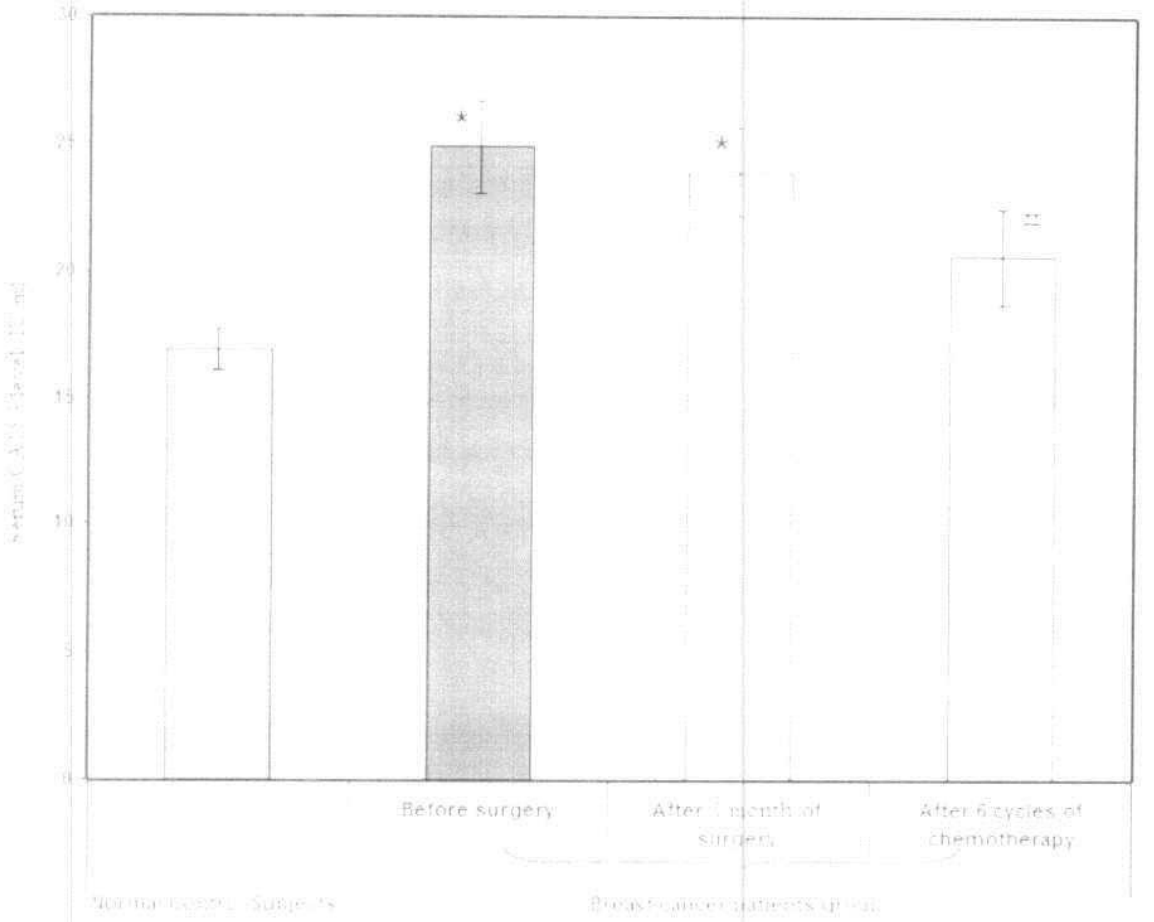

Figure 28: Bar chart representing the Mean ± S.E of CA15.3 (IU/ml) in normal control subjects and breast cancer patients before surgery, after one month of surgery and after 6 cycles of chemotherapy.

* : Significantly different from control group.
\# : Significantly different from breast cancer patients group before surgery.
Significance was considered at the level of P- value <0.05.

Correlations between the studied biochemical parameters and clinico-pathological parameters:

According to table (12) and (Figures 29-34), Bcl-2 showed significant inversely correlation with ER and Her-2 (P= 0.015, 0.045). Also P53 showed significant positive correlated with ER and significant inverse correlation with vascular invasion (P= 0.031, 0.004). Moreover, CA15.3 showed a significant positive correlation with either tumor size or clinical stage (P=0.01, 0.028).

Table (12): Correlations between the studied biochemical parameters and clinico-pathological parameters of the malignant group before surgery:

Clinico-pathological parameters		Biochemical parameters			
		Bcl-2 (ng/ml)	Bax (ng/ml)	P53 (U/ml)	CA 15.3 (IU/ml)
ER	r	-0.438	0.125	0.395	-0.144
	p	0.015*	0.510	0.031*	0.446
PR	r	-0.227	0.029	0.206	-0.197
	p	0.228	0.877	0.274	0.296
Her-2	r	-0.369	-0.089	0.000	-0.587
	p	0.045*	0.639	1	0.10
Tumor size	r	0.008	-0.165	0.024	0.464
	p	0.967	0.383	0.901	0.01*
lymph nodes involved	r	0.128	0.012	-0.172	-0.084
	p	0.501	0.950	0.363	0.659
Vascular invasion	r	-0.076	-0.164	-0.513	-0.155
	p	0.691	0.387	0.004*	0.412
Tumor pathological grade	r	-0.072	0.140	0.202	-0.36
	p	0.704	0.462	0.285	0.85
Clinical stage	r	0.156	-0.214	-0.093	0.408
	p	0.412	0.255	0.627	0.028*

r: Spearman coefficient * : Statistically significant at p <0.05

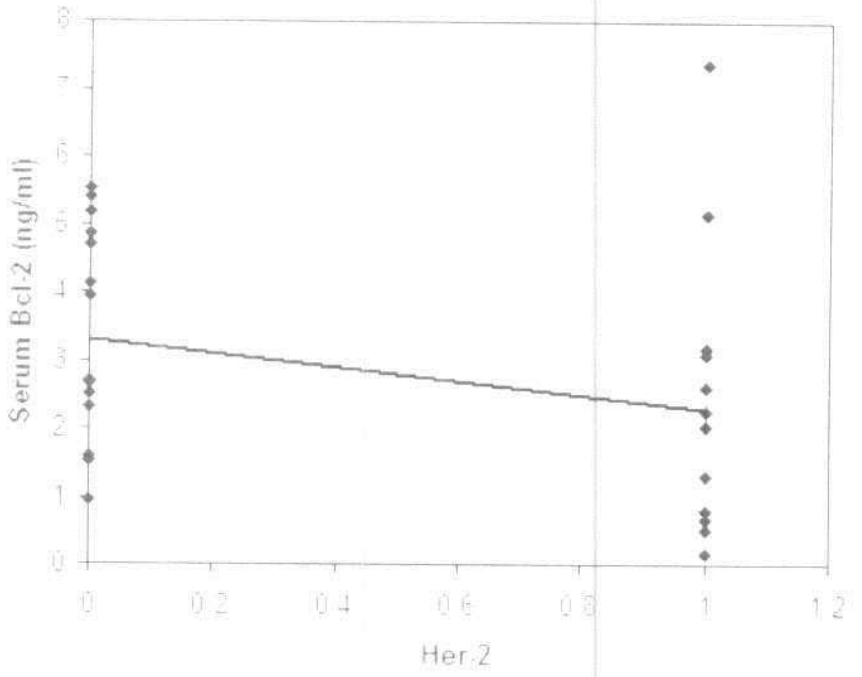

Figure 29: Correlations between Her-2 and Serum Bcl-2 (ng/ml).

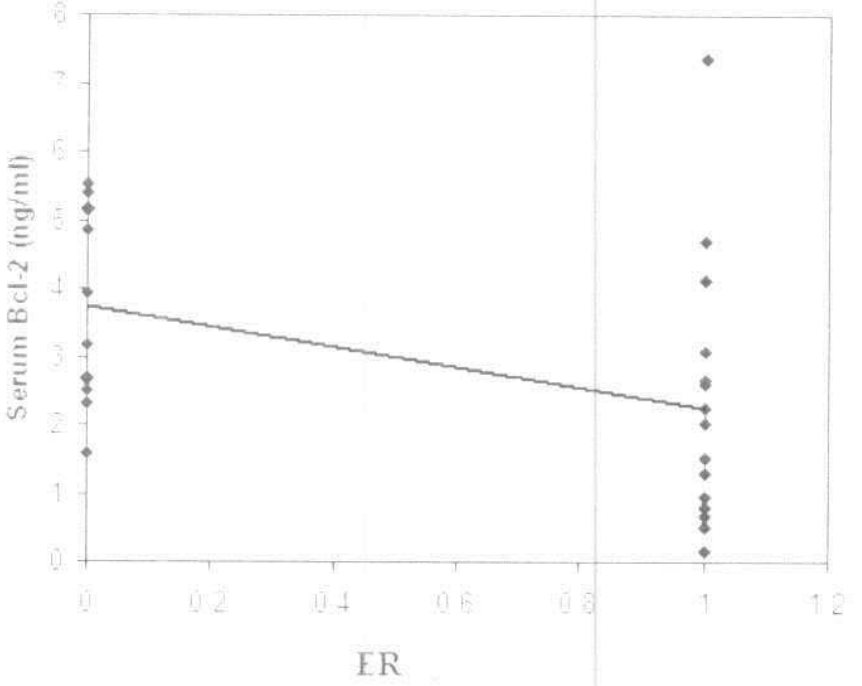

Figure 30: Correlations between ER and Serum Bcl-2 (ng/ml).

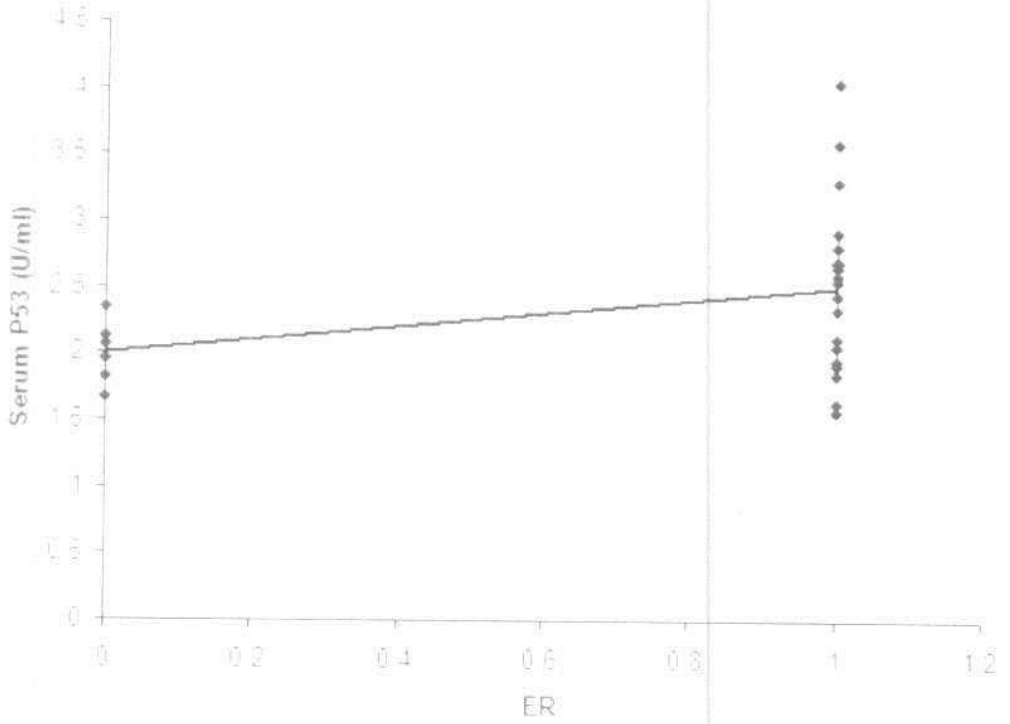

Figure 31: Correlations between ER and Serum P53 (U/ml).

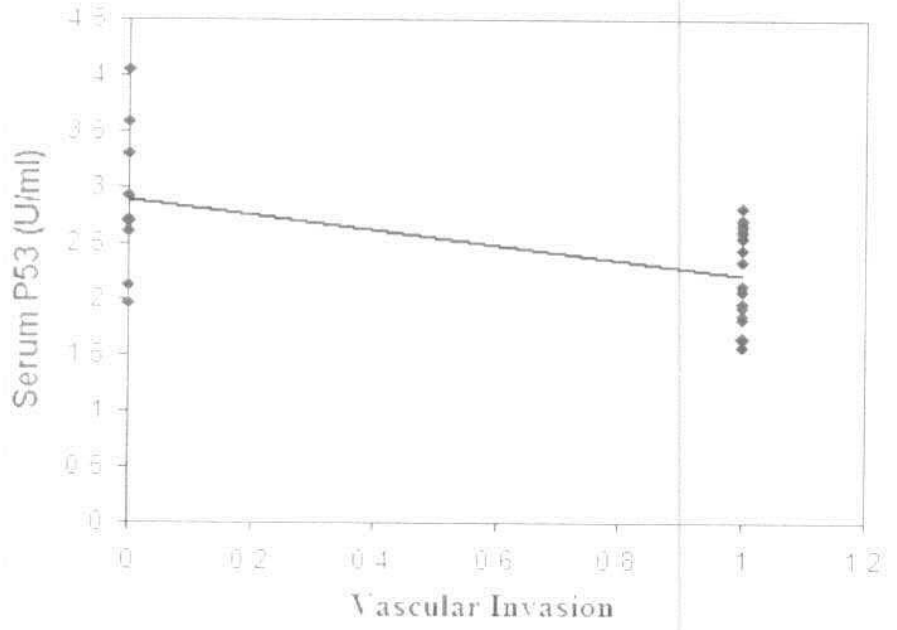

Figure 32: Correlations between vascular invasion and Serum P53 (U/ml).

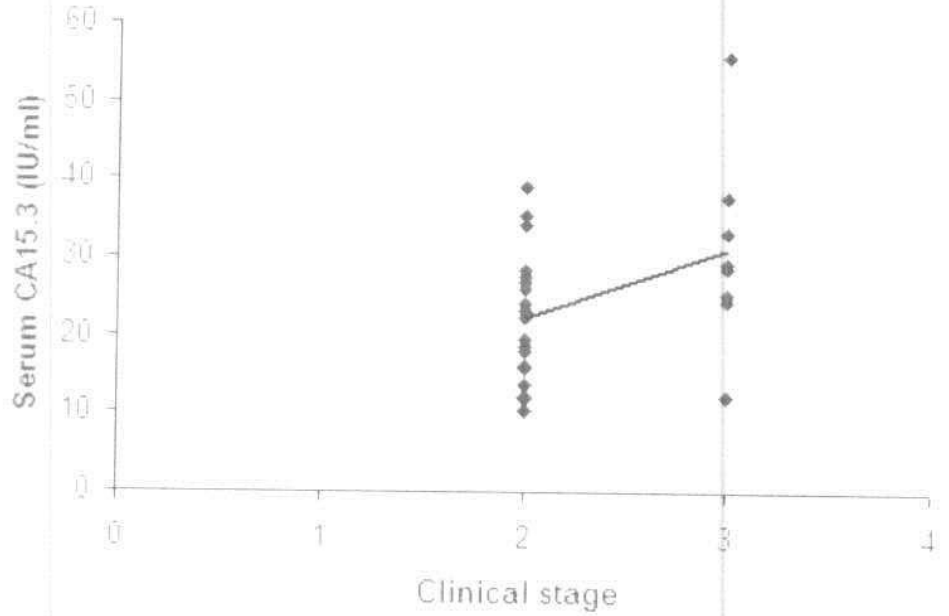

Figure 33: Correlations between tumor clinical stages and Serum CA15.3 (IU/ml).

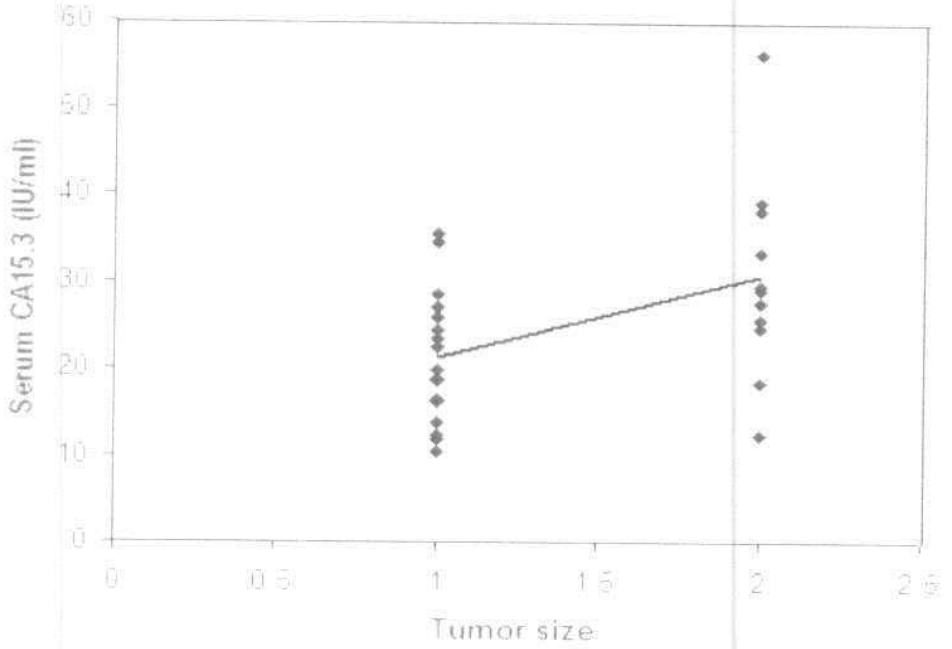

Figure 34: Correlations between tumor size and Serum CA15.3 (IU/ml).

Correlations between serum Bcl-2, Bax and P53 levels of malignant group before surgery, after one month of surgery and after 6 cycles of chemotherapy:

According to table (13) and (Figur35), P53 showed a significant inverse correlation with Bcl-2.

Table -13: Correlations between the serum P53, serum Bcl-2 and serum Bax levels of malignant group before surgery, after one month of surgery and after 6 cycles of chemotherapy

Parameter			Bcl-2 (ng/ml)	Bax (ng/ml)
P53 (U/ml)	Before surgery	r	-0.415	0.116
		p	0.023 *	0.381
	After 1 month of surgery	r	0.096	0.125
		p	0.614	0.512
	After 6 cycles of chemotherapy	r	0.089	0.069
		p	0.642	0.717

P*: < 0.05 was considered significant.

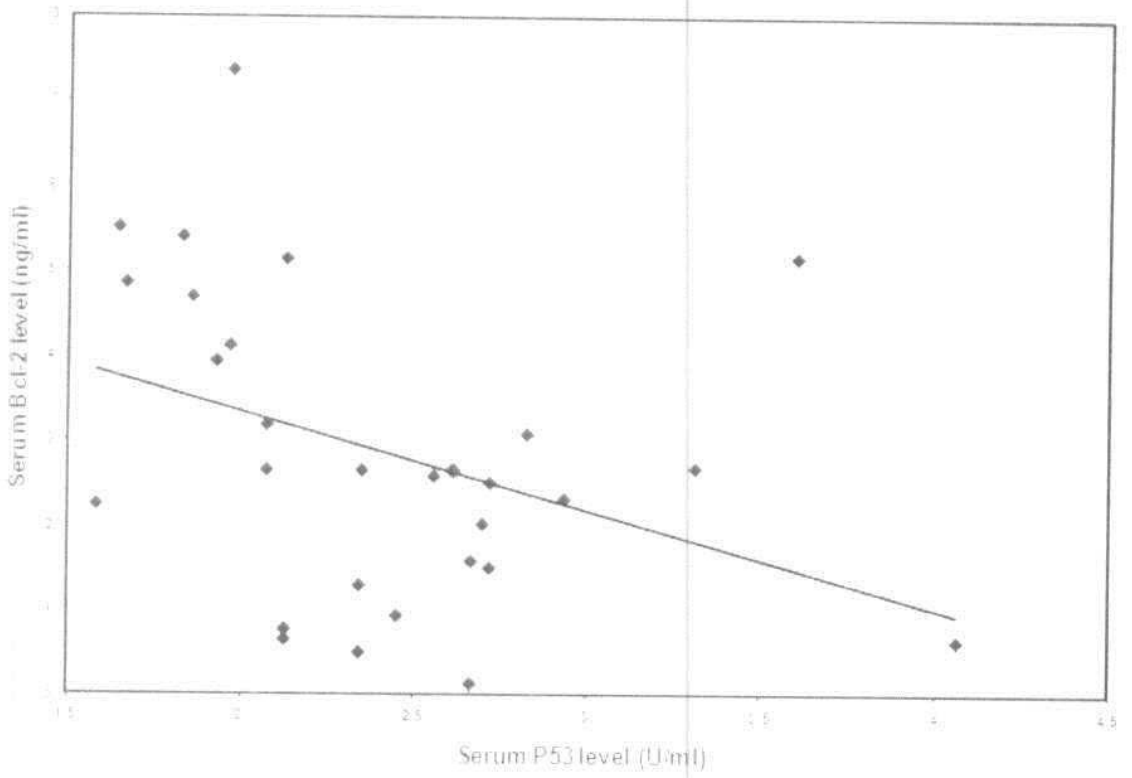

Figure 35: Correlation between the serum P53 and serum Bcl-2.

Comparison between the values of serum Bcl-2, P53 and CA15.3 as diagnostic markers in breast cancer patients using the receiver operating characteristic (ROC) curve analyses:

The ROC curve analysis was used to compare the diagnostic values of Bcl-2, P53 and CA15.3 depending on the area under the ROC curve (AUC). The higher AUC corresponds to a better diagnostic test. Serum Bcl-2 showed significant AUC (84.1), P< 0.001 with sensitivity (77%) and specificity (76%) at a cut off value (1.47). Serum P53 showed significant AUC (85.6), P < 0.001 with sensitivity (80%) and specificity (87%) at a cut off value (2.82).Serum CA15.3 showed significant AUC (76.1), P = 0.001 with sensitivity (47%) and specificity (100%) at a cut off value (25) table (14).

Based on the levels of serum Bcl-2, P53 and CA15.3 in breast cancer patients group, the ROC curves of the parameters were constructed as shown in (Figures 36&37).

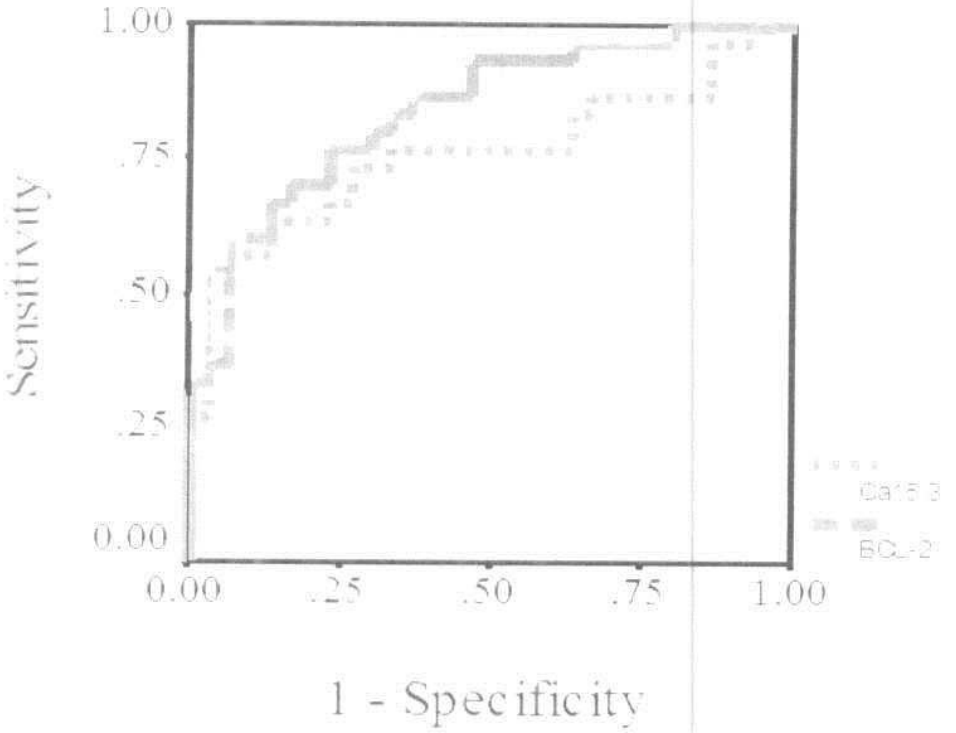

Figure 36: Graphical representation of the ROC curves for serum Bcl-2 and CA15.3 in breast cancer patients before surgery.

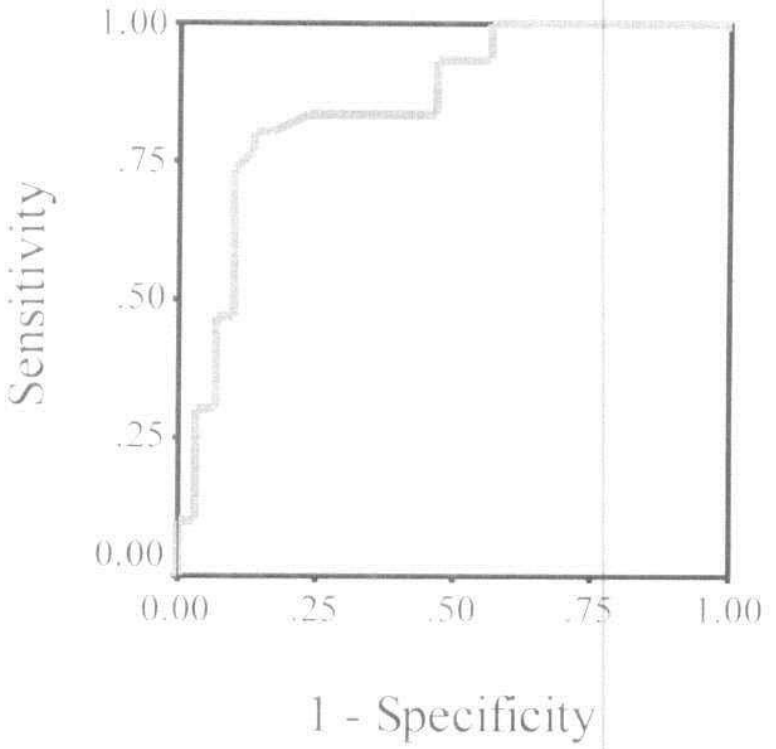

Figure 37: Graphical representation of the ROC curve for serum P53 in breast cancer patients before surgery.

Table (14): The ROC curve-based characteristics for serum Bcl-2, P53 and CA15.3 in breast cancer patients before surgery

Variables	Area under the curve (%)	Asymptomatic significance	Cut-off value	Sensitivity %	Specificity %
P53 (U/ ml)	85.6	<0.001*	2.82	80	87
Bcl-2 (ng/ ml)	84.1	<0.001*	1.47	77	76
CA 15.3 (IU/ml)	76.1	0.001*	25	47	100

* Significance was considered at P-value < 0.05.

Prognostic values of serum Bcl-2, P53 and CA 15.3 in breast cancer patients before surgery

To study the prognostic value of these parameters, the Kaplan Meir disease free survival (DFS) curves were constructed. As shown in Figure (38-40) Kaplan Meir Survival curves for breast cancer patients before surgery revealed that patients with elevated serum Bcl-2, P53 and CA15.3 higher than their corresponding cut off points were not statically different from those with low levels according log rank P= 0.34, 0.5 and 0.069 respectively.

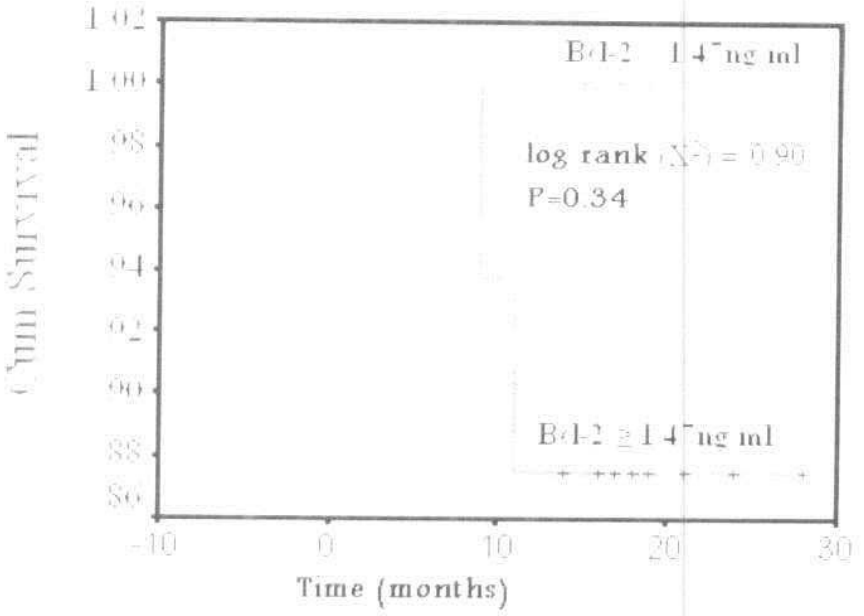

Figure 38: Kaplan Meir DFS of breast cancer patients before surgery in relation to serum Bcl-2.

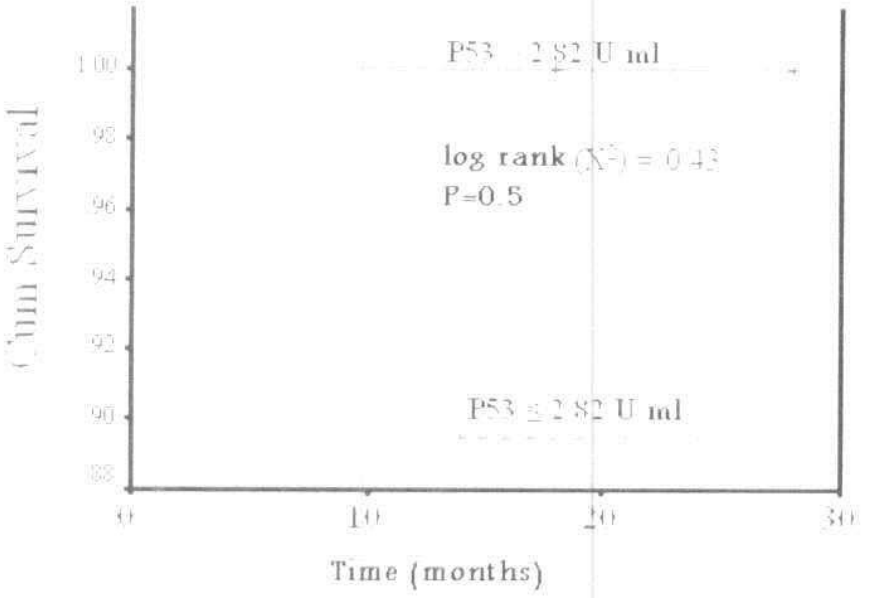

Figure 39: Kaplan Meir DFS of breast cancer patients before surgery in relation to serum P53.

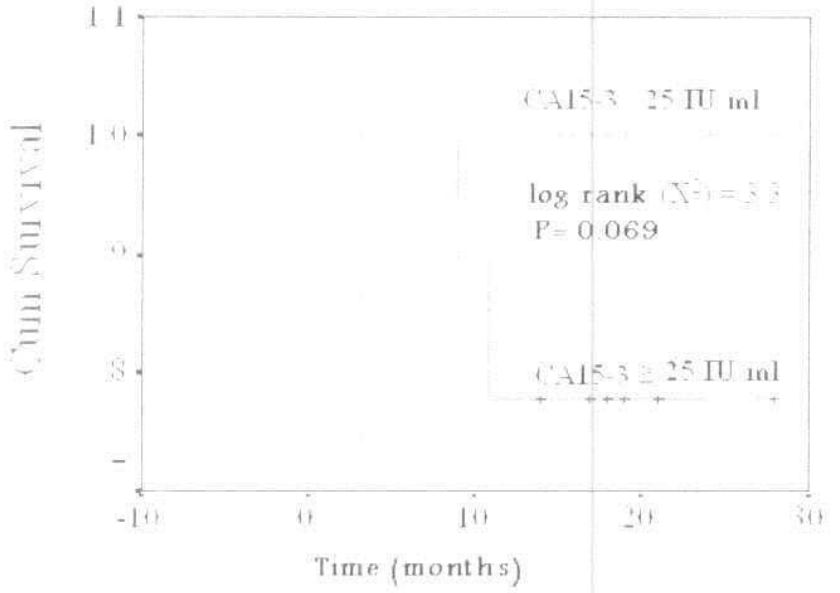

Figure 40: Kaplan Meir DFS of breast cancer patients before surgery in relation to serum CA15.3.

DISCUSSION

DISCUSSION

Breast cancer is the commonest cause of cancer death in women worldwide and invasive ductal carcinoma is the most frequent type; constituting up to 75% of cases. [177] It is an immensely heterogeneous disease, characterized by a broad variety of clinical development. The presence of molecular mechanisms affects tumor growth, proliferation, progression and metastatic potential. This limits significantly the prognostic value of TNM staging system therefore; various biomarkers are used as complement to the clinico-pathological staging in order to identify patients at higher risk of relapse (who need more aggressive systemic treatment). [178]

Biomarkers accepted for clinical use in breast cancer, such as CA 15.3, CEA and CA 27.29 have low sensitivity and specificity, and are thus more useful for patients at an advanced stage of breast cancer rather than for early cancer diagnosis. So, there is a need for new parameters to help in diagnosis and prognosis of primary breast cancer. [179]

Over the last several years, researches on the role of apoptosis in malignancy in general and in breast cancer in particular had increased. [180-183] Apoptotic markers are now being investigated and have a role in detecting the progression of cancer and its response to various chemotherapeutic agents. [184]

Apoptosis programmed cell death is an actively regulated cellular process, that leads to the destruction of individual cells. It plays a critical role in a variety of physiological processes during fetal development and in adult life and can be triggered by several stimuli such as radiation, drugs, and toxins. The apoptotic process is controlled by several genes including (P53, Bclx, Bax and Bak) and represses by (Bcl-2, BlxL and Mcl-1). The balance between expressions of these genes regulated the cell cycle and apoptosis. This balance is regulated by other stimuli such as P53 proteins or estrogen receptors in breast carcinomas. Excess of Bcl-2 promotes cell survival by inhibiting apoptosis, whereas excess of Bax accelerates cell death. [185] So in the present study, Bcl-2, Bax, P53 and CA15.3 were evaluated and correlated with clinico-pathological parameters.

Immunohistochemical staining is the most common method applied for evaluation of different Oncogenes. [186] In the present study, serum Bcl-2, Bax and P53 levels were estimated in serum using an Elisa technique. This technique allows for the easy quantification of Bcl-2, Bax and P53 levels, especially in serum. In addition, the serum assay for an oncoproteins can be easily and frequently performed because of its minimal invasiveness compared with surgically obtained tissue material. [186]

Bcl-2 is one protein which is able to block most proapoptotic stimuli, [187] and therefore promote cell survival. Bcl-2 family proteins regulate the intrinsic mitochondrial apoptosis pathway that responds to numerous stress stimuli, such as DNA damage or deprivation of growth factors. [188] When activated, this pathway leads to the permeabilization of the mitochondrial outer membrane and release of proapoptotic factors such as cytochrome c that activate the caspase cascade. [188] Prosurvival members, such as Bcl-2, maintain the integrity of mitochondria by preventing pro-apoptotic proteins such as Bax or Bak from initiating this pathway. [188] Although Bcl-2 prevents apoptosis, it also

inhibits proliferation; [86, 189, 190] these two processes involve independent pathways that are regulated differently. [191, 192]

In the present study, serum level of Bcl-2 was significantly higher in breast cancer patients before surgery than in normal healthy controls. These results agree with Mahdy et al 2011, [193] and Kallel-Bayoudh et al 2011, [194] who reported high level of Bcl-2 in breast cancer patients than normal controls. Previously it was reported that Bcl-2 was first discovered in B cells malignancies; [195] specific translocation moves Bcl-2 gene from its normal location at 18q21 into the locus at 14q32, resulting in the permanent activation of Bcl-2 gene and overproduction of Bcl-2 protein. [196] These studies may explain at least in part our findings.

The substantial increase of Bcl-2 among cancer cells points to a potentially critical role of this apoptosis suppressor protein in breast cancer progression. Overexpression of Bcl-2 protein may serve as determinator of an advantageous cell survival in breast tumor cells, ultimately leading to tumor progression and metastases. This hypothesis is supported by previous studies in experimental invivo model. [197, 198]

After one month of mastectomy and removal of the whole affected breast including the malignant mass, we expect that the level of Bcl-2 will be decreased than its value before surgery, but the comparative analysis showed insignificant difference between them. In view of these results, we can hypothesis two possible probabilities that may be responsible for maintaining the high level of Bcl-2: the first may be referred to the presence of free circulating malignant cells that already escaped from the primary tumor. The second may be due to incomplete clearance of this protein from the circulation.

The approach of chemotherapy for cancer patients was generally by intravenous administration. Drugs play their role by entering the blood circulation, therefore circulating cancer cells contact the chemotherapy drug. The results of our study demonstrated that the level of serum Bcl-2 level after 6 cycle of chemotherapy was statistically decreased than that after one month of surgery. It is well known that chemotherapeutic treatment significantly act on apoptosis and that interaction with Bcl-2 play a prominent role in this mechanism of action. [199] Therefore we can now convinced that this fact largely explain our findings reported in the present study.

However after completing six cycle of chemotherapy, patients included in this study were followed up clinically, radiological and laboratory for observation of any recurrence or metastasis.

On following the breast cancer patients for 24 months, two patients had metastasis, whereas 28 patients were free of metastasis. This result may lead us to predict that in at least some human cancer, Bcl-2 appear to promote metastasis. The development of metastasis and treatment failure of patients receiving the first line of chemotherapy could be explained by previously raised hypothesis that high serum Bcl-2 concentration may cause resistance to treatment by inhibiting apoptosis in cancer cell. [88] As some chemotheraptic regimens including FAC, induce apoptosis, protection from apoptotic cell death by Bcl-2 could prevent these drugs from working properly and perhaps these cells will be the source of metastasis.

In this study we found no significant association between serum Bcl-2 protein and disease free survival. Our results indicate that Bcl-2 was not independent prognostic marker.

Many reports disagree with our results as they found that high level of Bcl-2 was an independent prognostic factor for breast cancer patients [200-213] while other studies, as well as the present study, determined Bcl-2 protein expression to be not an independent significant prognostic factor. [200, 201] The determination of Bcl-2 protein expression as independent significant factor or not is due to the relationship between Bcl-2 protein expression and the other variables included in the multivariate models. [214]

The relationship between Bcl-2 and clinico-pathological characteristics had been investigated. We found that in spite of its role opposing tumor cell death; Bcl-2 is associated with biological features of the tumors which define a better prognosis, such as hormone receptors, Her-2 and mutant P53. This may in great part explain why Bcl-2 has been found to correlate with a better prognosis for breast cancer. It remains unclear why Bcl-2 would act as anti-apoptotic protein confers favorable prognosis out come. Several explanations have been put forward. For example, it has been demonstrated that the expression of Bcl-2 suppresses the proliferation of developing leukemia cells [215] by a cell cycle-inhibitory function, suggesting that overexpression of the Bcl-2 gene might delay the growth of cancer cells. Moreover, it has been suggested the Bcl-2 protein overexpression may have a proapoptotic function in some circumstances by increasing the half-life of Bax protein. [216]

Bax is one of the main effectors in breast cancer. However, in contrast with anti-apoptotic protein Bcl-2, there are relatively few clinical studies on the biological role of Bax in breast cancer. Bax expression was directly related to the expression of one of the main anti-apoptotic gene Bcl-2. This is not surprising, since it is well known that in breast cancer, the balance between the expressions of both genes is an important regulator of apoptosis. [80]

In the present study, it was found that serum Bax levels in breast cancer patients either before or after one month of surgery as well as after six cycle of chemotherapy were nearly within the same range and similar to the control value. This result is supported by histopathological finding previously performed by Vargas-Roig et al 2008. [217] who stated that intensity of nuclear Bax expression was weak or moderate in breast cancer cells and didn't change significantly after drug administration.

In addition we found no correlation between serum Bax level in breast cancer patients and the available clinico-pathological parameters of the tumors which could be tested. However the present study comprised of a relatively low number of patient and available tumor features were relatively few. Bax plays such a fundamental role in apoptosis that is hardly imaginable that it lacks any biological link to other basic molecular process in breast tumor cells. Putting this in mind, we must extend our study to a large number of samples.

The p53 tumor suppressor gene is an important negative regulator of cellular proliferation. The p53 gene product is induced in response to DNA damage. Evidence shows that the expression of p53 protein leads to cell cycle arrest in G 1 and in some cases to programmed cell death, or apoptosis. [218-220] Arrest of the cell cycle, presumably to

allow for DNA repair, as well as apoptosis, prevents the replication of damaged DNA and the subsequent propagation of genetic defects. This DNA replication control mechanism maintains fidelity in chromosomal transmission and has earned P53 the title "guardian of the genome." [220] Alterations in p53 lead to loss of its cell growth regulatory function, resulting in accelerated cell growth and increased DNA mutation frequency. The unchecked propagation of these mutations is thought to contribute to the development of human cancers. [221]

The result of the present study showed that serum P53 level was significantly lower in breast cancer patients than normal control group and correlated with estrogen receptors. In contrast, Nadasi et al 2007, [222] found that the level of P53 protein in breast cancer patients were up regulated and associated with estrogen receptors. He speculated that, positive ER in breast cancer patients forms complexes with the amino-terminus of P53 and protect it from degradation. Therefore, the low level of P53 observed in our study may be explained by relative small number of Positive cases. [223] As P53 is a tumor suppressor protein with proapoptotic properties, it is logic to have lower level of P53 in breast cancer patients compare with normal control which is the finding of the present study. However, no significant change was observed in serum P53 levels after one month of surgery and after six cycle of chemotherapy. This means that P53 protein has no role in monitoring the response of breast cancer patients to surgery and chemotherapy. [224]

In this study we found no significant association between serum P53 protein and disease free survival. These results agree with Gunel et al 2002, [225] who found no significant correlation between disease free survival and P53. In contrast Allred et al 1993, [226] found that P53 was the most significant independent predictor of reduced disease free survival.

The relationship between P53 and clinico-pathological characteristic had been investigated. We found that, P53 is correlated with estrogen receptors and negatively correlated with vascular invasion these finding may indicate that P53 is a favorable prognostic marker.

An inverse correlation between Bcl-2 and P53 in breast cancer had been reported in several studies, [227-232] our data confirmed a strong inverse relationship between Bcl-2 and P53 suggesting that the mutation or in activation of P53 are some how related to regulation of Bcl-2 in breast cancer. Consistent with these findings, Halder et al 1994, [233] stated that transfection of mutant P53 into P53 wild type breast cancer cell line suppresses Bcl-2. It is possible that the dual mechanism may be active at different times during the carcinogenic process, in which both Bcl-2 and P53 interfere with each other's regulatory process. [136]

Also, our study indicates that there is no relation between P53 and Bax. This result is supported with histopathological studies by Ali et al 2006, [227] who stated that mutation or inactivation of P53 in breast cancer patients being unable to promote gene expression after drugs administration.

CA15.3 is the mucin-1 marker that is the most widely used serum marker in breast cancer. [234] Currently, its main uses are in surveillances of patients with diagnosed disease and monitoring the treatment of patients with advanced disease. CA15.3 has been implicated in cell adhesion, immunity and metastasis compared with healthy breast tissue. [145, 235]

The evaluation of serum samples obtained from 30 women with breast cancer revealed significant higher CA15.3 levels than normal healthy women. At the same time, the present results showed that serum CA15.3 was significantly correlated with tumor size and clinical stage. These results indicate that high levels of CA15.3 appear to be related to tumor burden or imply the presence of malignant disease. These results supported the study of Hewala et al 2012, [221] Moreover, similar results were reported by Duffy 1999, [236] and Duggan et al 2004, [237] who stated that high preoperative CA15.3 predicts adverse outcome in patients with breast cancer.

After one month of mastectomy, we expect that the level of CA15.3 will be significantly decreased than its level before surgery but the comparative analysis showed insignificant decline between breast cancer patients' level of CA15.3 before and after one month of surgery and still significantly higher than control value. This result may be attributed to the long half life of CA15.3 previously reported by Kerin et al 1989, [238] who stated that, since CA15.3 has a long half life, a significant drop in CA15.3 antigen may not become obvious until three months after removal of the bulk mass of the tumor.

However, insignificant difference was observed in serum CA15.3 levels either after one month of surgery or after six cycle of chemotherapy. This means that serum CA15.3 protein has no role in monitoring the response of breast cancer patients to surgery and chemotherapy. Although many studies [239] reported that serum CA15.3 play a role in monitoring the response of breast cancer patients to therapy, the absence of this role of CA15.3 in the present study may be due to small sample size including in this study. In addition we can attribute the significant higher level of CA15.3 than normal control value to the toxic effect of the drug on normal epithelia and rebound neutrophilia. [240]

The significant elevation in Serum levels of Bcl-2 and CA15.3 and the significant decline in the serum level of P53 in breast cancer patients before surgery compared to normal controls suggest the possibility of using anyone of these parameters for diagnosis of breast cancer, to differentiate the breast cancer patients from normal healthy controls. This directed us to compare the diagnostic values of these parameters to determine which of them has the highest and lowest diagnostic value. This comparison also involved determination of the precise cut-off value and the corresponding sensitivity and specificity for each parameter. This comparison was carried out using the ROC curve analysis in such a way that the greater area under the ROC curve corresponds to a better diagnostic test. Serum P53 showed the greater area under the curve (85.6%) followed by Bcl-2 (84.1%), then CA15.3 (76.1%).

The sensitivity, specificity and cut-off value for diagnosing breast cancer patients were 80%, 87% and 2.82% U/ml for P53; 77%, 76 and 1.47 ng/ml for Bcl-2 and 47%, 100% and 25 IU/ml for CA15.3. These results suggest that serum P53 is superior to Bcl-2 and CA15.3 for diagnosis of breast cancer patients. Although serum P53 and Bcl-2 protein have been observed in breast cancer patients, to the best of our knowledge, this is the first study that compares the diagnostic value of serum P53 and Bcl-2 protein with those of serum CA15.3 with determination of the precise cut-off value, sensitivity and specificity of each protein in breast cancer patients.

SUMMARY

SUMMARY

In the present study, we evaluated the diagnostic, prognostic and follow up values of serum Bcl-2, Bax and P53 in comparison with serum CA15.3 in breast cancer patients. All of these parameters were measured in serum samples collected from normal healthy control group (n=30) and breast cancer females of recently detected breast carcinoma of clinical stages II and III (n 30), before surgery, one month following surgical removal of the breast and after 6 cycles of FAC chemotherapy. Serum Bcl-2, Bax and P53 levels were measured in all studied groups by ready-to-use Enzyme linked immunosorbent assay (ELISA) kits. Serum CA15.3 was measured using a ready-for-use Immunoradiometric assay (IRMA) kit.

The serum levels of serum Bcl-2 of breast cancer patients either before or after one month of surgery as well as after 6 cycle of chemotherapy were significantly higher than those of control group. It was also noticed that serum Bcl-2 level of breast cancer patients which was significantly similar to those after one month of surgery, significantly decreased after 6 cycles of chemotherapy. On comparing the level of this parameter after 6 cycles of chemotherapy with those after one month of surgery, the difference was significant. These results indicated that, serum Bcl-2 level may be considered as a diagnostic biomarker in breast cancer and can be used as a useful marker in the follow up chemotherapeutic treatment. Our results showed a significant negatively correlation of serum Bcl-2 with estrogen receptors, Her-2 and P53 in breast cancer patients group. This means that, serum Bcl-2 can be valuable in predicting breast cancer progression.

The level of serum Bax in breast cancer patients either before or after one month of surgery as well as after 6 cycles of chemotherapy were nearly within the same range and insignificantly less than those of control group. In addition we found no correlation between serum Bax level in breast cancer patients and the available clinic-pathological parameters of the tumors which could be tested.

The levels of serum P53 of breast cancer patients either before or after one month of surgery as well as after 6 cycle of chemotherapy were significantly less than those of control group. It was also noticed that serum P53 level of breast cancer patients was significantly similar to those after one month of surgery and significantly decreased after 6 cycle of chemotherapy. On comparing the level of this parameter after one month of surgery and those after 6 cycle of chemotherapy the difference was not significant.

According to the results of the present study, the serum levels of CA15.3 were significantly higher in breast cancer patients than the controls. We found no follow up role for serum CA15.3 in patients with primary breast cancer neither following the surgical removal of the breast nor after 6 cycles of chemotherapy. The serum CA15.3 showed a significant correlation with clinical stage and tumor size in breast cancer patients.

In the present study, it was found that serum Bcl-2, P53 and CA15.3 were diagnostic markers. On comparing their diagnostic values to determine which one is superior to the other, the ROC curve analysis was applied. Inspection of the curves showed that the ROC curve for serum P53 was above ROC curves for Bcl-2 & CA15.3, respectively. The area under the curve was 85.6% for P53, 84.1%for Bcl-2 and 76.1% for CA15.3. The optimum

cut-off value selected for serum P53 was 2.82 U/ml at which the sensitivity was 80% and the specificity was 87%, the optimum cut-off value selected for serum Bcl-2 was 1.47 ng/ml at which the sensitivity was 77% and the specificity was 76% and the optimum cut-off value selected for serum CA15.3 was 25 IU/ml at which the sensitivity was 47% and the specificity was 100%. Hence serum P53 is superior to Bcl-2 & CA15.3, respectively, as a diagnostic tumor marker in breast cancer patients.

Up to the best of our knowledge, this is the first study comparing the diagnostic values of these three parameters using the ROC curve analysis showing P53 as the most superior diagnostic marker. Bcl-2 was also found to have a role in monitoring the effect of surgery & chemotherapy on breast cancer patients.

Due to the small sample size of patients in this study, we need to verify the results obtained from our study by using larger sample size of patients and longer time of Follow up.

CONCLUSIONS

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From this study, we could conclude that:

1) Serum Bcl-2 is a diagnostic biomarker in breast cancer patients with estrogen receptors and Her-2 and can be used in the follow up of the breast cancer patients after chemotherapeutic treatment.

2) CA15.3 can be used as diagnostic markers for breast cancer patients with clinical stages II & III.

3) No role for serum Bax in breast cancer patients either before or after the surgical removal of the breast or after chemotherapy.

4) P53 is superior to Bcl-2 & CA15.3 as a diagnostic tumor marker in breast cancer patients with clinical stages II & III.

5) Bcl-2, P53 and CA15.3 were not independent prognostic markers.

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PROTOCOL

دراسة البروتينات بى سى ال-٢، باكس وبى ٥٣ كمؤشرات بيولوجية في مصل دم مريضات سرطان الثدي

Study of serum Bcl-2, Bax and P53 proteins as biological markers in breast cancer patients

خطة بحث مقدمة الى

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الماجستير فى الكيمياء الطبية التطبيقية

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BACKGROUND

Breast cancer is the most common malignancy affecting women. So there is need for new markers to monitor the effect of treatment and predict the future status of the patient for successful management. (1)

Normal breast development is controlled by a balance between cell proliferation and apoptosis, and there is strong evidence that tumor growth is not just a result of uncontrolled proliferation but also of reduced apoptosis. The balance between proliferation and apoptosis is crucial in determining the overall growth or regression of the tumour in response to chemotherapy, radiotherapy and hormonal treatments. All of these act in part by inducing apoptosis. (2)

The Bcl-2 (B-cell lymphoma-2) family of genes encodes proteins that may promote or inhibit apoptosis. Proapoptotic proteins include Bax, Bak, Bad, and Bcl-xs whereas Bcl-2 and Bcl-xL are antiapoptotic. The Bcl-2 gene was first identified as a part of the fusion gene in t (14; 18) translocation commonly found in follicular lymphoma. (3) In humans, Bcl-2 protein is expressed in about 80% of breast cancers and is correlated with the expression of estrogen and progesterone receptors—good prognostic features in breast cancer. This surprising association between an apoptosis inhibitor and good prognostic features is confirmed by the improved survival of patients with tumors that are Bcl-2 positive compared with those that are Bcl-2 negative. (4) In patients with metastatic breast cancer, the serum level of Bcl-2 was significantly elevated than in normal healthy controls and was also found to be correlated with breast cancer clinicopathological parameters. (5) In patients with advanced ovarian carcinoma, serum levels of Bcl-2 were found to be significantly higher in cancer patients than in normal healthy controls but with no prognostic value. (6)

Bax is a proapoptotic member of the Bcl-2 family. Bax expression is induced by γ radiation and chemotherapeutic drugs. Bcl-2 protein can inhibit the mitochondrial apoptotic pathway via binding to

its proapoptotic protein Bax, thereby preventing the release of cytochrome c and the activation of apoptotic cascade. [7] For apoptosis to occur, the concentration of Bax protein should be greater than that of Bcl-2 protein. Kim [8] notified that a reduction in Bax was associated with a poor response to chemotherapy in metastatic breast cancer.

Apoptosis is a defined form of cell death, which plays an important role in the development of multicellular organisms and in the regulation and maintenance of the populations in tissues under physiological and pathological conditions. [9] The Bcl-2/Bax ratio may serve as a potential molecular marker for prediction of tumor prognosis. [10] It has been suggested that the Bax/Bcl-2 ratio may be more important than either parameter alone in determining apoptosis. [11] Ghanem et al [12] demonstrated that alterations of the Bcl-2/Bax balance may influence the clinical outcome of nephroblastoma patients by deregulation of programmed cell death.

P53 is a tumor suppressor gene known as the guardian of the genome. [13] The product of th P53 gene is a nuclear phosphoprotein expressed in normal human cells. In the serum of health subjects,the presence of P53 protein product is extremely rare. Mutations in this gene cause a accumulation of non-functional proteins due to a longer half-life of several hours. The clinic implications of the development of serum P53 protein in cancer patients are unknown whether t presence of the P53 protein is associated with poor prognosis or if it has early diagnostic value. Mutation or over expression of P53 protein has been observed in up to 52% of primary bre cancers. [15] In a case-control study of breast cancer patients and normal healthy controls, the se level of P53 protein was found to be a significant risk factor for breast carcinoma. [16]

CA15.3 (cancer antigen 15.3) is one of the most reliable tumor markers used in monito breast cancer patients. [17] CA15.3 is a circulating breast cancer-associated antigen. [18] Preoper measurements of CA15.3 levels are of little value in the early detection of breast cancer and thu not be used for diagnosing early breast cancer. [19]

AIM OF THE STUDY

This work aims to study serum Bcl-2, Bax and P53 proteins as biomarkers and their correlations with serum CA-15.3 and clinicopathological parameters in breast cancer patients.

SUBJECTS AND METHODS

Subjects:

This study will include 60 females divided into 2 groups:

Group I:

It will include 30 premenopausal females of recently detected breast carcinoma of clinical stages II and III [20]. Patients will be selected from those admitted to Surgery Department and Cancer Management and Research Department, Medical Research Institute, University of Alexandria. All patients will be subjected to surgery (modified radical mastectomy) [21], followed by adjuvant chemotherapy, consisting of 6 cycles of intravenous FAC (5-Fluorouracil 500 mg/m^2, Adriamycin 50 mg/m^2 and cyclophosphamide 500 mg/m^2) [22].

Group II:

It will include 30 healthy females as control group of matched age, menstrual cycle & socioeconomic status as patients.

Exclusion criteria: all individuals suffering from immunological, liver diseases, other malignancies, history of chemotherapy or radiotherapy.

Methods:

All patients in group I will be subjected to radiological investigations (Ultrasonography of liver and plain X-ray chest), CT scan and bone scan when needed. After surgery and chemotherapy they will be re-evaluated clinically, radiologically to estimate the clinical response. Patients will be clinically followed up for at least 1 year.

Laboratory investigations:

All patients in group I will be subjected to routine laboratory investigations: complete blood count [23], liver and renal function tests [24].

All biochemical tests will be carried out as follows:-

1-Blood samples will be collected once from control group (group II), while three samples will be obtained from group I before surgery, one month after surgery and after 6 cycles of chemotherapy. Blood will be withdrawn to separate serum in which biochemical analysis will be performed.

2-Serum Bax (Ray Biotech, USA), Bcl-2 (eBioscience, Germany) [25] and P53 (eBioscience, Germany) [26] levels will be measured in all studied groups using ready-for-use ELISA kits according to manufacturer's protocol.

3- Serum CA15.3, a standard tumor marker in breast cancer patients, will be measured using a ready-for-use immunoradiometric assay (IRMA) kit according to manufacturer's protocol (Biosource, USA). [27]

Histopathological studies:

In group I, estrogen (ER) and progesterone (PR) receptors status, number of axillary lymph node involvement, tumor size, tumor grade and vascular invasion will be evaluated and correlated with serum Bcl-2, Bax, P53 and CA15.3 levels.

Statistical Analysis

Statistical analysis will be preformed using (SPSS 11.5 Software).

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ARABIC SUMMARY

الملخص العربي

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في هذه الدراسة، قمنا بتقييم القيم التشخيصية والروية المستقبلية والمتابعة لقيم كل من بي سي ال-٢و باكس و بي ٥٣ في مصل الدم ومقارنتهم مع الانتيجين السرطاني ١٥-٣ في مريضات سرطان الثدي المرحلة الاكلينيكية الثانية والثالثة. تم قياس كل من هذه المعاملات في عينات مصل الدم التي تم تجميعها من السيدات الصحيحات (المجموعة الضابطة) (وعددهن ٣٠) و السيدات المريضات بسرطان الثدي (وعددهن ٣٠). وتم قياس هذه المعاملات قبل اجراء العملية الجراحية و بعد مرور شهر من الاستئصال الجراحي للثدي و بعد ست دورات من العلاج الكيميائي المركب. وقد استخدمت طريقة القياس المناعي الانزيمي المعدة للاستخدام مباشرة. تم قياس مستوى الانتيجين السرطاني ١٥-٣ في مصل الدم في مريضات سرطان الثدي و المجموعة الضابطة باستخدام طريقة القياس المناعي الاشعاعي المعدة للاستخدام مباشرة.

لوحظ ارتفاع مستوي بي سي ال-٢في مصل دم مجموعة مريضات سرطان الثدي بالمقارنة بالمجموعة الضابطة سواء قبل الجراحة او بعد مرور شهر من الجراحة وكذلك بعد اعطاء ست جرعات من العلاج الكيميائى المركب. لوحظ ارتفاع ذو دلالة احصائية بالمقارنة بالمجموعة الضابطة، وكذلك لا يوجد اختلاف احصائي في معدله قبل الجراحة وبعد مرور شهر من الجراحة وعلي العكس وجد تناقص ذو دلالة احصائية في معدله بعد العلاج الكيميائى بالمقارنة بمعدله قبل الجراحة. كما انه عند مقارنة معدله بعد الجراحة وبعد العلاج الكيميائى لم نجد اى دلالة احصائية. ويمكن ان نستخلص ان بي سي ال-٢ في مصل دم مريضات سرطان الثدي يمكن استخدامه كدليل حيوى في تشخيص ومتابعة مريضات سرطان الثدي.

وقد اظهرت الدراسة ان هناك ارتباط احصائى سلبى بين بي سي ال-٢ في مصل الدم لمريضات سرطان الثدي وبين مستقبلات هرمون الاستروجين ،مستقبلات الهير-٢ و بى ٥٣ وهذا يعنى ان بي سي ال-٢ في مصل الدم يمكن ان يستخدم في التنبو بتقدم سرطان الثدى.

وعلي العكس وجد ان مستوي باكس في مصل دم مريضات سرطان الثدي كان تقريبا يعادل مستوي قياسه في المجموعة الضابطة سواء قبل الجراحة ، بعد شهر من الجراحة وبعد ست دورات من العلاج الكيميائى المركب. وكذلك اثبتت الدراسة انه لا يوجد اي ارتباط بين باكس في مصل الدم والدلالات الاكلينيكية الباثولوجية.

اظهر قياس مستوي بى ٥٣ في مصل الدم لمريضات سرطان الثدي انخفاض ذو دلالة احصائية ملحوظة سواء قبل الجراحة، بعد شهر من الجراحة وبعد ست دورات من العلاج الكيميائى المركب عن مستوي قياسه في المجموعة الضابطة. وكذلك اظهرت الدراسة انخفاض مستوى قياس بي٥٣ في مصل دم مريضات سرطان الثدى ذو دلالة احصائية بعد ست دورات من العلاج الكيميائى المركب عنه قبل الجراحة. بينما لا توجد علاقة ذو قيمة احصائية بين مستوي قياسه قبل وبعد اجراء الجراحة.

وقد اظهرت الدراسة ان هناك ارتباط احصائى بين بي ٥٣ في مصل الدم لمريضات سرطان الثدي وبين مستقبلات هرمون الاستروجين ،وكذلك ارتباط احصائي سلبي بين بى٥٣وغزو الأوعية الدموية وهذا يعني ان بي ٥٣ في مصل الدم يمكن ان يستخدم في التنبو بتقدم سرطان الثدى.

وفقا لنتائج هذه الدراسة، زاد مستوي الانتيجين السرطاني ١٥-٣ بشكل ملحوظ في مصل دم مجموعة مريضات سرطان الثدي قبل الجراحة بالمقارنة بالمجموعة الضابطة. يشير هذا الى ان الانتيجين السرطاني ١٥-٣ قد يلعب دور في تطور سرطان الثدي الأولي و ايضا امكانية استخدامه كدليل تشخيصي مع انخفاض الحساسية للكشف عن سرطان الثدي في مراحله المبكرة. دور الانتيجين السرطاني ١٥-٣ في متابعة مريضات سرطان الثدي الأولى لم يلاحظ سواء بعد الإستئصال الجراحي أو بعد ست دورات من العلاج الكيميائي. توجد علاقة وثيقة بين المرحلة الاكلينيكية و حجم الورم و مستوى الانتيجين السرطاني ١٥-٣ في مريضات سرطان الثدي.

في هذه الدراسة، وجد ان كلا من بي ٥٣،بي سي ال-٢ والانتيجين السرطان ١٥-٣ في مصل الدم دلائل تشخيصية. تم تطبيق التحليل باستخدام منحني الـ ROC لمقارنة القيم التشخيصية لتحديد ايهم افضل من الاخر. وجد ان منحني ROC للـ بي ٥٣ اعلى من منحنيات ROC لكل من بي سي ال-٢ و الانتيجين السرطاني ١٥-٣. كانت المساحة تحت المنحنى ٨٥.٦% لـ بي ٥٣ ، ٨٤.١% ل بي سي ال-٢و ٧٦.١% للانتيجين السرطاني ١٥-٣. قيمة المعيار المثالية التي تم اختيارها للـ بي ٥٣ هي ٢.٨٢ وحدة/ملي و كانت الحساسية عندها هي ٨٠% و الخصوصية

٨٧% و قيمة المعيار المثالية ل بي سي ال-٢ هى ١.٤٧ نانو جرام/ملي و كانت عندها الحساسية ٧٧% و الخصوصية هي ٧٦% و قيمة المعيار المثالية ل الانتيجين السرطان ١٥-٣ هي ٢٥ وحدة عالمية/مل و كانت عندها الحساسية هي ٤٧% و الخصوصية هي ١٠٠%. و بالتـــــالي فان الـ بي ٥٣ كدليل أورام تتخصصي في مريضات سرطان الثدي هو الأفضـــل من بي سي ال-٢ و الانتيجين السرطاني ١٥-٣.

يصل الي حد علمنـــا، ان هذه هي أول دراسة تقارن القيم التشخيصية لهذه المعايير الثلاثة باستخدام تحليل منحنى ROC وتظهر ان بي ٥٣ هو الدليل التشخيصي الافضل. ووجـــد ايضا ان الـ بي ٥٣ و بي سي ال-٢ له دور في متابعة تاثير الجراحة والعلاج الكيميائي علي مريضات سرطان الثدي.

الاستنتاج

من هـــذه الدراسة، يمكننا ان نستنتج مـــا يـــلي:

بي سي ال-٢ يستخدم كدليل حيوي لتشخيص سرطان الثدي بالاستعانة ب مستقبلات هرمون الاستروجين ومستقبلات الهير -٢ وكذلك يمكن استخدامه لمتابعة المريضات بعد نهاية العلاج الكيميائي. بينما الانتيجين السرطاني ١٥-٣ في مصل الدم يمكن استخدامه كدليل لمريضات سرطان الثدي في المرحلة الاكلينيكية الثانية والثالثة وعلي العكس اظهر الباكس انه ليس له اى دور في متابعة مريضات سرطان الثدي. وكذلك وجد ان بي ٥٣ يتفوق عل ال بي سي ال-٢ والانتيجين السرطاني ١٥-٣ كدليل للاورام في مريضات سرطان الثدي في المرحلة الاكلينيكية الثانية والثالثة.

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دراسة البروتينات بى سى ال-٢، باكس وبى ٥٣ كموشرات بيولوجية في مصل دم مريضات سرطان الثدي

مقدمة من

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بكالوريوس العلوم في الكيمياء و الكيمياء الحيوية
جامعة الإسكندرية، ٢٠٠٧

للحصول على درجة

الماجستيـــر

فى

الكيميـاء الطبيـة التطبيقيـة

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دراسة البروتينات بى سى ال-٢ ، باكس وبى ٥٣ كموشرات بيولوجية في مصل دم مريضات سرطان الثدي

رســـــالـة علمية

مقدمة الى معهد البحوث الطبية- جامعة الإسكندرية
استيفاء للدراسات المقررة للحصول على درجة

المـاجستيـــر

فى

الكيميـــاء الطبيـــة التطبيقيـــة

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