Study of big endothelin-1 as a tumor marker in Egyptian female patients with breast cancer

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By

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B.Sc.in Chemistry and Biochemistry, Faculty of Science,
Alexandria University, 2004
Diploma of Biochemistry, Faculty of Science,
Alexandria University, 2005

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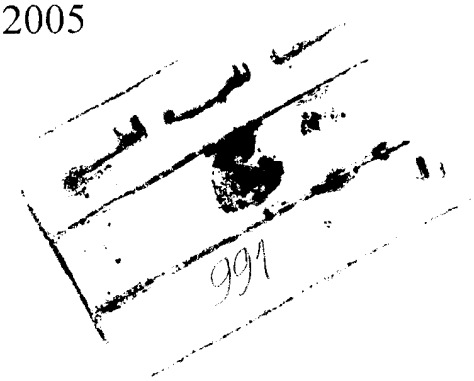

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LIST OF ABBREVIATIONS

A	: **A**driamycin.
AC	: **A**driamycin and **c**yclophosphamide.
ADAM17	: **A** **d**isintegrin **a**nd **m**etalloproteinase**17**.
AIs	: **A**romatase **i**nhibitors.
AJCC	: **A**merican **J**oint **C**ommittee on **C**ancer.
ASCO	: **A**merican **S**ociety of **C**linical **O**ncology.
ATM	: **A**taxia **t**elangiectasia **m**utated.
AUC	: **A**rea **u**nder **c**urve.
BC	: **B**reast **c**ancer.
Bcl-2	: **B**-**c**ell **l**ymphoma-**2**.
Big ET-1	: **B**ig **e**ndothelin-**1**.
BMDCs	: **B**one **m**arrow **d**erived **c**ells.
BRCA1	: **B**reast **ca**ncer susceptibility gene**1**.
BRCA2	: **B**reast **ca**ncer susceptibility gene**2**.
BRIP1	: **BR**CA1**i**nteracting **p**rotein c-terminal helicase**1**.
BSE	: **B**reast **s**elf-**e**xamination.
C	: **C**yclophosphamide.
CA15.3	: **C**arbohydrate **a**ntigen**15.3**.
CAF/FAC	: **C**yclophosphamide, **A**driamycin and 5-**f**luorouracil.
CBE	: **C**linical **b**reast **e**xamination.
CEF	: **C**yclophosphamide, **e**pirubicin and 5-**f**luorouracil.
CHEK2	: **Ch**eck point **k**inase**2**.
CMF	: **C**yclophosphamide, **m**ethotrexate and 5-fluorouracil.
CTRL	: **Con**trol.
D	: **D**ocetaxel.
DCIS	: **D**uctal **c**arcinoma ***insitu***.

DDR : **D**NA **d**amage **r**esponse.

DES : **D**iethyl**s**til**b**estrol.

DNA : **D**eoxyribo**n**ucleic **a**cid.

DSBs : **D**ouble **s**trand **b**reaks.

E : **E**pirubicin.

EC : **E**pirubicin and **c**yclophosphamide.

ECE-1, 2 : **E**ndothelin **c**onverting **e**nzyme-**1, 2**.

EGFR : **E**pidermal **g**rowth **f**actor **r**eceptor.

EGTM : **E**uropean **G**roup on **T**umor **M**arker.

EIA : **E**nzyme **i**mmuno**a**ssay.

ER : **E**strogen **r**eceptor.

ETs : **E**ndothelin**s**.

ET-1 : **E**ndothelin-**1**.

ETR : **E**ndothelin **r**eceptor.

ET_AR : **E**ndothelin $_A$ **r**eceptor.

ET_BR : **E**ndothelin $_B$ **r**eceptor.

F : 5-**f**luorouracil.

FAD : **F**ood and **D**rug **A**dministration.

FEC : 5-**f**luorouracil, **e**pirubicin and **c**yclophosphamide.

FNA : **F**ine **n**eedle **a**spiration.

GPCR : **G**-protein **c**oupled **r**eceptor.

GRB-2 : **G**rowth factor **r**eceptor **b**ound protein-**2**.

GSK3β : **G**lycogen **s**ynthase **k**inase-**3β**.

HER-2 : **H**uman **e**pidermal growth factor **r**eceptor-**2**.

HIF-1α : **H**ypoxia **i**nducible **f**actor-**1α**.

HRP : **H**orse**r**adish **p**eroxidase.

HRT : **H**ormone **r**eplacement **t**herapy.

ICAM-1 : **I**ntercellular **a**dhesion **m**olecule-**1**.

IDC : **I**nvasive **d**uctal **c**arcinoma.

IL-10 : **I**nter**l**eukin-**10**.

IRMA : **I**mmuno**r**adio**m**etric **a**ssay.

LCIS : **L**obular **c**arcinoma ***insitu***.

LMCs : **L**ow and **m**iddle income **c**ountries.

LN : **L**ymph **n**ode status.

M : **M**ethotrexate.

MBC : **M**ale **b**reast **c**arcinoma.

MLH : **M**ut **l** **h**omologs.

MMP : **M**atrix **m**etallo**p**roteinase.

MRI : **M**agnetic **r**esonance **i**maging.

MSH2 : **M**ut **s** **h**omologs**2**.

MT-MMP1 : **M**embrane **t**ype **m**atrix **m**etallo**p**roteinase**1**.

MUC : **M**ucin.

NBS1 : **N**ijmegen **b**reakage **s**yndrome gene**1**.

P53 : Tumor suppressor **p**rotein**53**.

PALB2 : **P**artner **a**nd **l**ocalizer of **B**RCA**2**.

PARP-1 : **P**oly **A**DP-**r**ibose **p**olymerase-**1**.

PDGFR-β : **P**latelet **d**erived **g**rowth **f**actor **r**eceptor-**β**.

PKC-δ : **P**rotein **k**inase **c**-**δ**.

PLC-γ : **P**hospho**l**ipase **C**-**γ**.

PO : **P**rophylactic salpingo-**o**ophorectomy.

PR : **P**rogesterone **r**eceptor.

PTEN : **P**hosphatase and **ten**sin homologue.

PTS : **P**roline, **t**hreonine and **s**erine domain.

R : Spearman coefficient.

RNA : **R**ibo**n**ucleic **a**cid.

ROC : **R**eceiver **o**perating **c**haracteristic.

SD : Standard deviation.

SERMs : Selective estrogen receptor modulators.

STD : Standard.

TAC : Docetaxel, adriamycin and cyclophosphamide.

TAMs : Total associated macrophages.

TGF-β : Transcription growth factor-β.

TMB : 3, 3′, 5, 5′ tetra methyl benzidine.

TNM : Tumor size, lymph node and metastasis.

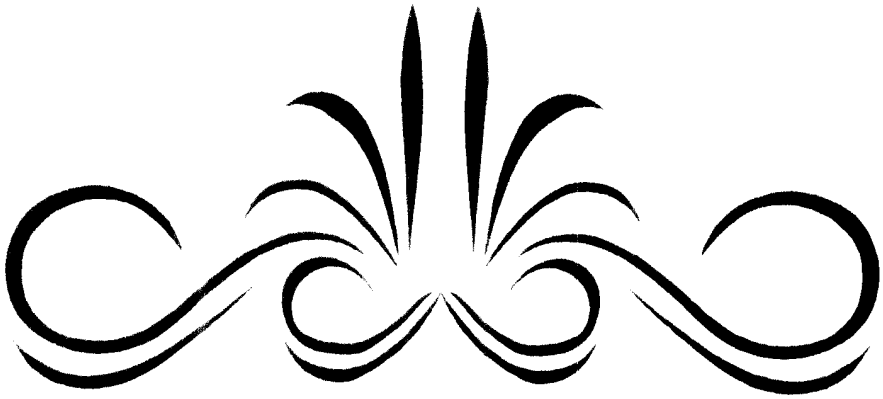
TSGs : Tumor suppressor genes.

VEGF : Vascular endothelial growth factor.

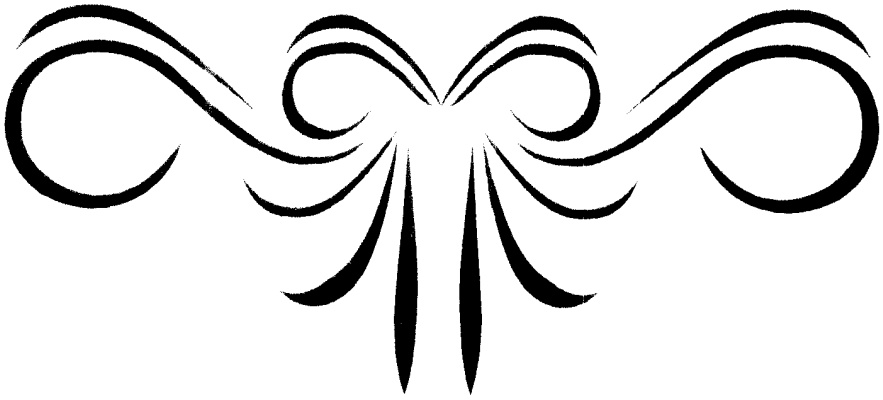
VNTR : Variable number of tandem repeats.

VSMC : Vascular smooth muscle cell.

ZAP-70 : Zeta chain associated protein kinase-70.



Introduction



INTRODUCTION

Cancer is a cluster of diseases involving alterations in the status and expression of multiple genes that confer a survival advantage and undiminished proliferative potential to somatic or germinal cells [1].

Alterations primarily in three main classes of genes, (proto) oncogenes, tumor suppressor genes and DNA (deoxyribonucleic acid) repair genes collectively contribute to the development of cancer genotype and phenotype that resists the natural and inherent death mechanism(s) embedded in cells (apoptosis and like processes), coupled with dysregulation of cell proliferation events, DNA damage response (DDR) and usually accompanied by an extended chromosomal instability [2–4]. There is increasing evidence to suggest that cancer is also driven by 'epigenetic changes' like DNA methylation and altered patterns of histone modifications, leading to alterations in chromatin condensation status thereby regulating expression of certain set of specific genes[5,6].

Breast cancer

Breast cancer (BC) refers to cancers originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk [7]. Breast cancers are traditionally classified based on their histopathology and stage. Most of the breast cancers (95%) are adenocarcinomas, which are further divided into *in situ* carcinomas and invasive carcinomas. The *in situ* carcinomas, which comprise approximately 15-30% of all breast carcinomas, can be classified further into either ductal or lobular carcinomas *in situ* (DCIS or LCIS, respectively), and they may develop into invasive breast cancers [8]. In turn, invasive carcinomas include numerous different subtypes such as ductal carcinomas, lobular carcinomas and tubular carcinomas (Figure 1) [9].

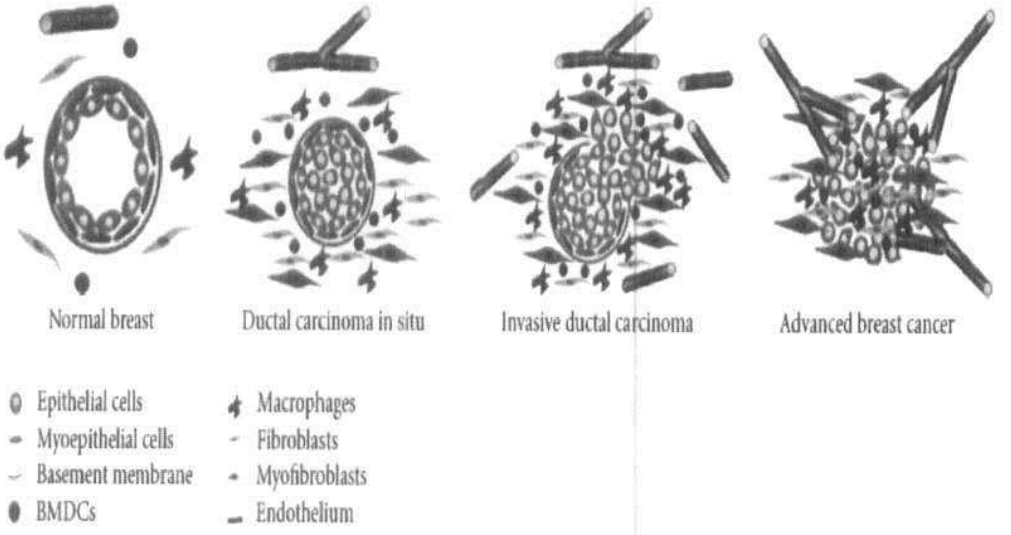


Figure (1): Schematic presentation of breast cancer progression accompanied with stromal cells [9]. Normal breast duct is composed of epithelial cells and a layer of myoepithelial cells separated from the stroma by a basement membrane. Stromal cells include fibroblasts, bone marrow derived cells (BMDCs), endothelial cells, and other cells. DCIS is associated with luminal epithelial cells proliferation, recruitment and expansion of stromal cells. In invasive ductal carcinoma (IDC), the myoepithelial cell layer is degraded with the underlying basement membrane and cancerous cells invade the surrounding microenvironment. Advanced breast cancer is associated with loss of myoepithelial cell layer and basement membrane, invasion of epithelial cells, proliferation of stromal cells, and angiogenesis.

Epidemiology of breast cancer:

Breast cancer is the most common cancer affecting women worldwide. Globally, about 1.3 million new cases are diagnosed annually accounting for 23% of the total cancers cases and leading cause of cancer death accounting for 14% of all cancer deaths among females [10, 11]. The incidence is highest in developed countries, but it is also increasing at alarming rates in low- and middle-income countries (LMCs), where it is overtaking cervical cancer as the most frequent malignancy diagnosed in women. Mortality from breast cancer has decreased significantly in developed countries over the past few decades as a result of earlier diagnosis and wide availability of multimodality therapy [11].

Approximately 2% of breast cancers occur in young women between 20and34 years of age [12] (Table 1). After diagnosis, young women with breast cancer, aged<35 years, are more likely to suffer from recurrence and death than older women [13].

Table (1): Incidence of breast cancer by age [14].

Age	Annual incidence/100.000women
<20	0.1
20-24	1.4
25-29	8.1
30-34	24.8
35-39	58.4
40-44	116.1
45-49	198.5

Although women in Egypt (and in LMCs generally) have a lower risk of breast cancer than do their counterparts in high-income countries, young women in Egypt (and other LMCs) are at a higher risk today than their mothers and grandmothers were at the same age, [15-17] which is consistent with a classic birth-cohort effect.

Risk factors of breast cancer:

Patients with breast cancer belong to one of three groups:

a. Sporadic breast cancer (75%): Patients without family history or those who have a breast biopsy with proliferative changes.

b. Cluster family breast cancer (20%): Relevant history of breast cancer in the family and breast biopsy with proliferative breast changes with no association with mutations.

c. Genetic mutation breast cancer (5%): Women with a genetic predisposition and related to mutations in the breast cancer susceptibility gene 1 (BRCA1) and breast cancer susceptibility gene 2 (BRCA2) [18].

Both non-genetic and genetic factors are involved in the etiology of breast cancer. Non-genetic factors include exposure to female reproductive hormone (both endogenous and exogenous), increassing age, body mass index, alcohol intake, benign breast disease and physical activity. The genetic component of the disease is reflected on a tendency to cluster in families, although this could also reflect shared life-style and environment [19, 20] (Table 2) [21].

I- Non-genetic risk factors:

a) Age:

The incidence of BC increases substantially with age, with more than 300 cases per100, 000 women per year between the ages 30–60 years. [22]

b) Gender:

In contrast to the breast cancer in women, male breast carcinoma (MBC) is rare, accounting for less than 1% of all cases of breast carcinoma with an incidence of 1 in 100.000 men [23,24].

c) Family history:

Family history contributes an increased risk when the relatives are close (first degree relatives include mother, sister, or daughter), there are multiple family members (including second degree relatives), if family members were premenopausal at the time of diagnosis (or less than age 50), or if there were any cases of bilateral breast cancer[25,26]. Women with a family history of breast cancer in a first degree relative that was postmenopausal have a slightly increased risk with a relative risk of 1.5 times the general public . A woman with a first degree family member that was premenopausal at the time of diagnosis (or age less than 50) has a relative risk of 3.0 times the general public [25,27].

d) Hormonal factors

i) Endogenous hormones:

The development of breast cancer in many women appears to be related to female reproductive hormones. Epidemiologic studies have consistently identified a number of breast cancer risk factors associated with increased exposure to endogenous estrogens. Early age at menarche, nulliparity or late age at first full term pregnancy, and late age at menopause increase the risk of developing breast cancer [20].

ii) Exogenous hormones:

When a pregnant woman uses diethylstilbestrol (DES), an orally active synthetic estrogen because it was thought to lower their chances of spontaneous abortion, has an increased risk of breast cancer. Risk is also increases in a female Childs who were exposed in utero [28, 29].

Several studies suggested that with ever use of oral contraceptives associated with a very small increase in the relative risk of localized breast cancer. The greatest risk was observed among current and recent users (within 4 years of diagnosis), with the risks

declining with increasing time since last use. No increased risk was apparent for women who had discontinued use 10 or more years ago [30].

In postmenopausal women, the use of combined estrogen and progestin hormone replacement therapy (HRT), associated with increased breast cancer risk [20].

e) Dietary and lifestyle factors

i) Alcohol:

Alcohol has also been consistently associated with an increased risk of BC [31]. Decreased intake of nutrients such as vitamin c, folate, and β-carotene may enhance the risk related to alcohol consumption [20].

ii) Diet:

There is a large international variation in breast cancer incidence, with countries with high fat diets having higher rates of breast cancer than those with diets lower in fats, suggested that high fat intake might be associated with increased breast cancer risk [20].

iii)Obesity:

Is associated with both an increased risk of breast cancer development in postmenopausal women and increased breast cancer mortality [20].

iv)Tobacco smoking:

There is limited evidence with inconsistent results suggesting that tobacco smoking is associated with female breast cancer risk, in particular when smoking starts early, and before a woman's first full-term pregnancy (before the breast tissue matures) and continues for several decades [32,33].

v) Physical activity:

Several studies suggested that physical activity protects against premenopausal and probably postmenopausal breast cancer [34].

vi)Lactation:

There is a suggested protective effect of lactation (i.e. breastfeeding) against both pre and postmenopausal breast cancer especially for long-term lactation at a young age [34]. Lactation delays the return of ovulation after pregnancy and is associated with a different hormonal milieu (increased prolactin), which results in less estrogen exposure to the breast[30].

f) Benign breast disease

Benign breast lesions are classified as proliferative or nonproliferative. Nonproliferative disease is not associated with an increased risk of breast cancer, whereas proliferative disease without atypia results in a small increase in risk. Proliferative disease with atypical hyperplasia is associated with a greater risk of cancer development [20].

g) Breast density

Mammographic breast density has emerged as an important predictor of breast cancer risk. A significant component of breast density is genetically determined, although density has also been shown to vary with the initiation and discontinuation of postmenopausal HRT [20].

h) Environmental factors

Exposure to ionizing radiation increases breast cancer risk, and the increase is particularly marked for exposure at a young age. A markedly increased risk of breast cancer development has been reported in women who received mantle irradiation for the treatment of Hodgkin's lymphoma before age 15 years. Other environmental factors, including exposure to electromagnetic fields and organochlorine pesticides, have been suggested to increase breast cancer risk [20].

II- Genetic risk factors:

a) Mutations in BRCA1 and BRCA2:

Mutations in BRCA1 and BRCA2 genes are associated with a significant increase in the risk of breast and ovarian carcinoma and account for 5% to 10% of all breast cancer. Cells that lack BRCA1 or BRCA2 are unable to sense DNA damage properly, transmit and process the damage response signal, or repair DNA damage by homology directed recombination [20]. In cells with defective BRCA1 and/or BRCA2 repair of DSBs (Double Strand Breaks) occurs using alternative, error-prone repair pathways [35].One of those alternatives routes is the base excision repair mechanism [36]. This pathway usually repairs single-strand breaks. Inhibition of this pathway increases the number of unrepaired single-strand breaks, which leads to collapsed replication forks and produces DSBs. Poly (ADP-ribose) polymerase 1 (PARP-1) is a nuclear protein with a zinc-finger DNA binding domain that links to the site of the DNA damage. Binding of PARP-1 to DNA initiates its activity adding Poly (ADP-ribose) polymers to itself as well as to the surrounding histones [37]. (Figure 2)[38]

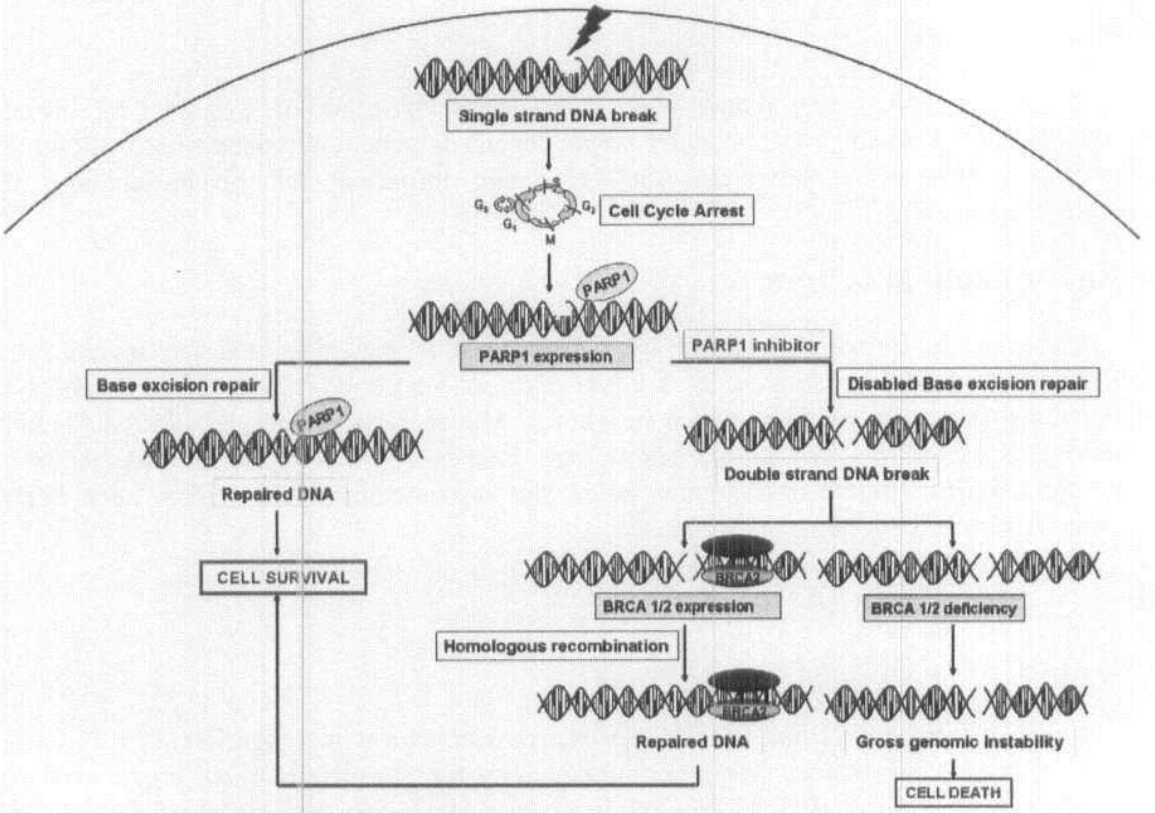


Figure (2): Mechanisms of DNA repair in tumors lacking BRCA1 and BRCA2 compared with non-transformed cells [38].

b) Mutations in other genes:

Other tumor suppressor genes (TSGs) that are associated with rare familial breast cancer such as tumor supressor protein p53 (P53), phosphatase and tensin homologue (PTEN), and ataxia telangiectasia-mutated (ATM). Additional low to moderate risk genes such as check point kinase2 (CHEK2), BRCA1 interacting protein C-terminal helicase1 (BRIP1), partner and localizer of BRCA2 (PALB2), nijmegen breakage syndrome gene (NBS1), DNA repair protein RAD50 and the mismatch repair genes mut S homologs (MSH2) and mut L homologs (MLH) [39].

Table (2): Risk Factors for Breast Cancer [21].

Risk Factor	Relative Risk
BRCA1 or *BRCA2* mutation	10.0–32.0
Family history of cancer (no known mutation)	
1 first-degree relative	1.5–2.0
2 first-degree relatives	3.0
3 or more first-degree relatives	4.0
1 second-degree relative	1.2–1.5
Therapeutic radiation to chest at <30 yr. of age	7.0–17.0
Hormonal factors	
Late (age >30 yr.) parity or nulliparity	1.2–1.7
Early (age <12 yr.) menarche or late menopause (age >55 yr.)	1.2–1.3
Combined hormone-replacement therapy (e.g., for 10 or more yr.)	1.5
Postmenopausal obesity	1.2–1.9
Alcohol consumption (2 drinks/day vs. none)	1.2
Smoking before first live birth	1.2
Sedentary lifestyle	1.1–1.8
White race	1.1–1.5
Breast density (very dense vs. mainly fatty)	5.0
Atypical ductal or lobular hyperplasia or lobular carcinoma in situ on previous breast biopsy	4.0

Staging of breast cancer:

The stage of a cancer is one of the most important factors in selecting treatment options, and it uses the Tumor size, lymph Nodes and Metastasis (TNM) system. When a patient's T, N, and M categories have been determined then this information is combined in a process known as stage grouping to determine a woman's disease stage. This is expressed as from Stage 0 (the least advanced stage) to Stage IV (the most advanced stage)[40]. Breast cancer staging helps clinicians estimate prognosis also help acquire data on the effectiveness of various interventional strategies, including screening[41]. The American Joint Committee on Cancer (AJCC) TNM staging is illustrated in (Table 3, 4) [40].

Table (3):TNM Staging [40]

Classification	Definition
Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
TIS	Carcinoma in situ
T1	Tumor up to 2 cm in greatest dimension
T2	Tumor > 2 cm and < 5 cm in greatest dimension
T3	Tumor > 5 cm in greatest dimention
T4	Tumor of any size with direct extension to chest wall or skin, only as described below
T4a	Extension to chest wall, not including pectoralis muscle
T4b	Edema (including peau d'orange) or ulceration of the skin of the breast, or satellite skin nodules confined to the same breast
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed (e.g., previously removed)
N0	No regional lymph node metastasis
N1	Metastasis in movable ipsilateral axillary lymph node(s)
N2	Metastasis in ipsilateral axillary lymph nodes fixed or matted, or in clinically apparent ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastasis
N2a	Metastasis in ipsilateral axillary lymph nodes fixed to one another (matted) or to other structure
N2b	Metastasis only in clinically apparent ipsilateral internal mammary nodes and in the absence of clinically evident axillary lymph node metastasis
N3	Metastasis in ipsilateral infraclavicular lymph node(s), or in clinically apparent ipsilateral internal mammary lymph node(s) and in the presence of clinically evident axillary lymph node metastasis; or metastasis in ipsilateral supraclavicular lymph node(s) with or without axillary or internaly mammary lymph-node involvement
N3a	Metastasis in ipsilateral infraclavicular lymph node(s) and axillary lymph node(s)
N3b	Metastasis in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
N3c	Metastasis in ipsilateral supraclavicular lymph node(s)
Distant Metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table (4): TNM stage grouping for breast cancer [40]

Stage grouping	TNM Classification		
0	Tis	N0	M0
I	T1a	N0	M0
IIA	T0	N1	M0
	T1a	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	N0	M0
	T4	N1	M0
	T4	N2	M0
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

Screening of breast cancer:

Early detection of breast cancer plays the leading role in reducing mortality rates and improving the patients prognosis [42]. Various screening practices such as clinical breast examination (CBE), ultrasonography, mammography, and breast self examination (BSE) have been recognized [43].

The benefits of screening for breast cancer can be summarized as follows [44]:

1. The detection of breast cancer or a pre-malignant lesion in a woman who was previously unaware of the existence of lesion.
2. The successful treatment of that cancer or precancerous condition and prevention of death from breast cancer.
3. The reassurance that when screening tests are truly negative, there is comfort in knowing that a breast cancer or premalignant condition is unlikely to be present.

Breast cancer screening program, including monthly breast self-exam, annual or semi-annual clinical breast exam, and annual screening mammography is recommended[20,45]. Women with a history of radiation exposure should be screened with a combination of mammography and magnetic resonance imaging (MRI) [45].

Mammogram

Mammography is the most common method of breast imaging. It uses low-dose amplitude-X-rays to examine the human breast [46]. Screening mammography reduces breast cancer mortality for women ages 50 to 69 by 15% to 30% over 15 years or longer [43, 46].

Magnetic resonance imaging (MRI)

Breast MRI screening has been recommended for high risk women with BRCA1/2 mutation carriage. The sensitivity of the breast MRI and cancer detection rates within this group are better than with mammography [47].

Ultrasonography

Screening ultrasonography has been reported to result in up to a 30% absolute increase in the detection of invasive cancer in women with dense breasts, for whom the sensitivity of mammography is reduced and the risk of cancer is increased [48, 49].

Breast self examination (BSE)

BSE is by far the quickest, easiest and most cost effective method available for the detection of breast cancer. There are no studies that prove BSE decreases mortality this only applies to palpable lesions, but for those women who are too young for mammography and/or who have no family history and would therefore not be identified as elevated risk patients [50].

Clinical breast examination (CBE)

CBE is necessary for detection and evaluation of the 8% to 17% of breast cancers not seen by mammograms [51, 52]. Women should undergo CBE every 3 years between the ages of 20 and 39 years, and annually after age 40 years. This exam should take place during periodic health examinations [53].

Symptoms of breast cancer:

General symptoms are breast lumps or lump in the armpit which is hard possess uneven edges and usually does not hurt. Change in the size, shape or feel of the breast or nipple. Secretion of fluid from the nipple will be bloody, clear to yellow or green color and look like pus. Symptoms of advanced breast cancer may be includes bone pain, breast pain or discomfort, skin ulcers, swelling of one arm (next to the breast with cancer) and weight loss [54].

Diagnosis of breast cancer:

A biopsy remains the standard technique for diagnosing both palpable and non-palpable breast abnormalities. The available biopsy techniques for the diagnosis of

palpable breast masses are fine needle aspiration (FNA), core cutting needle biopsy, and excisional biopsy. Non-palpable lesions can be biopsied with image-guided core needle biopsy or surgical excision after wire localization [20]. FNA is easily performed, rapid and painless but requires a trained cytopathologist for accurate specimen interpretation and does not reliably distinguish invasive cancer from ductal carcinoma in situ [20].

Core cutting needle biopsy has many of the advantages of FNA, but provides a histologic specimen suitable for interpretation by any pathologist. In addition, estrogen and progesterone receptor (ER and PR) status and presence of human epidermal growth factor receptor 2 (HER-2) over expression can be routinely available [20]. When excisional biopsy is performed for diagnosis; a small margin of grossly normal breast should be excised around the tumor [20].

Treatment of breast cancer:

The treatment options for early stage breast cancer include local-regional and systemic therapy. Surgery and radiotherapy can minimize the risk of local recurrence, while systemic adjuvant chemotherapy and hormonal treatment is related to prolongation of survival rate [55]

Surgery

Surgery is the most common treatment for breast cancer. Several types of surgery are practiced. An operation to remove the breast (or as much of the breast as possible) is mastectomy. An operation to remove the cancer but not the breast is called breast-sparing surgery or breast-conserving surgery. Lumpectomy and segmental mastectomy (also called partial mastectomy) are types of breast-sparing surgery. They usually are followed by radiation therapy to destroy any cancer cells that may remain in the area. In most cases, the surgeon also removes lymph nodes under the arm to help determine whether cancer cells have entered the lymphatic system and gone beyond the breast. [56]

Radiotherapy

Radiation therapy uses high-energy radiation to shrink tumors and kill cancer cells and stop them from growing [56, 57].The rays may come from radioactive material outside the body and be directed at the breast by a machine (external radiation). The radiation can also come from radioactive material placed directly in the breast in thin plastic tubes (implant radiation) [56].

Systemic therapy

Systemic therapy uses anti-cancer drugs that are injected into a vein or given by mouth. These drugs travel through the bloodstream to all parts of the body. Systemic therapy includes biologic therapy, chemotherapy, and hormone therapy. Systemic treatment given to patients before surgery is called neoadjuvant therapy. It is often used to shrink the tumor enough to make surgical removal possible or allow for less extensive surgery. This may allow women otherwise needing mastectomy to undergo breast-conserving surgery. Neoadjuvant therapy has been found to be as effective as therapy given after surgery in terms of survival, disease progression, and distant recurrence. Systemic treatment given to patients after surgery is called adjuvant therapy [58].

a) Biologic therapy

In fifteen to twenty-five percent of BC, there is an overexpression of HER-2 protein in the cancer cells, indicating a high risk of recurrence. Trastuzumab (Herceptin) is a humanized monoclonal antibody against HER-2 protein. When given concomitantly or after adjuvant chemotherapy for 12 months as adjuvant treatment for early HER-2 positive BC, trastuzumab reduced the risk of recurrence by approximately 50% and the risk of death by approximately 30% [59, 60].

Another drug, Lapatinib, has been found to be effective in delaying disease progression in women with HER-2 positive, advanced breast cancer who have become resistant to trastuzumab. A new generation of anti-HER-2 targeted therapies is currently in development [61].

b) Chemotherapy

Adjuvant treatment is administered to a large majority of patients, according to prognostic factors including age, nodal status, tumor size, histological grade, hormone-receptor status and expression of HER-2 [62].

Clinical studies have shown that chemotherapy can be benefit to women with node positive and node negative breast cancer, with tumors that are either hormone receptor positive or negative, regardless of age or menopausal status [20].

Chemotherapy for breast cancer consisting of multiple cycles of polychemotherapy is well established as an important strategy for lowering the risk of breast cancer recurrence and improving survival. The drugs may be given orally or parenterally. Either way, chemotherapy is a systemic therapy because the drugs enter the blood stream and circulate throughout the body in order to reach cancer cells. However, this means that chemotherapy also harms cells that divide rapidly under normal circumstances, such as cells in the bone marrow, digestive tract, and hair follicles. Chemotherapy is given in cycles: a treatment period following by a recovery period. It has substantial side effects, including fatigue, nausea, vomiting, myelosupression, neuropathy, diarrhea, and alopecia [20, 56]. A large number of chemotherapy agents and combinations are effective in treatment of breast cancer (Table 5) [20, 63-65].

Table (5): Commonly used chemotherapeutic agents and regimens in breast cancer patients

Drug class	Agent(s) in class	Combination regimens
Anthracyclines	Doxorubicin (Adriamycin) (A), Epirubicin (E)	Alkylating agents / anthracyclines + / - antimetabolites regimens (such as AC, EC, CEF, CAF, FEC, FAC, CMF,TAC)
Taxanes	Paclitaxel, Docetaxel (D), albumin nano-particle bound paclitaxel	Anthracyclines / taxanes regimens (such as doxorubicin/ Paclitaxel or doxorubicin/ docetaxel)
Alkylating agents	Cyclophosphamide (C)	Docetaxel/ Capecitabine Gemcitabine/ Paclitaxel
Antimetabolites	5-fluorouracil (F), Capecitabine, Methotrexate (M), Gemcitabine	Taxanes/ platinum regimens (such as Paclitaxel/ Carboplatin or docetaxel/ Carboplatin)
Vinca alkaloids	Vinorelbine,Vinblastine	
Platinum salts	Cisplatin, Carboplatin	

Clinical trials have demonstrated advantages for multiple cycles (four to eight) of chemotherapy compared to single cycle regimens. Multiple individual trails also showing superiority of anthracycline and/or taxanes based chemotherapy compared to CMF based non anthracycline regimens [20].

c) Hormone therapy

Aromatase inhibitors (AIs) such as anastrazole, letrozole and exemestane are now part of standard treatment for most postmenopausal women with estrogen receptor-positive and/or progesterone-receptor-positive early invasive breast cancer[66].AIs function through inhibition of the aromatase enzyme that converts androgens into estrogen [67].

Selective Estrogen Receptor Modulators (SERMs) are a class of compounds that act on the estrogen receptor. SERMs play a key role in breast cancer chemoprevention. These agents antagonize estrogens in some tissues and mimic their action in others. For example, tamoxifen and toremifene act as estrogen antagonists in breast tissue and as estrogen agonists in the endometrium. Conversely, raloxifene behaves as an estrogen antagonist in both the breast and the endometrium [68].Tamoxifen administered for duration of 5 years result in a 41% reduction in the annual rate of breast cancer recurrence and a 34% reduction in the annual death rate for women with ER positive breast cancer [20].

Bilateral prophylactic salpingo-oophorectomy (PO)

(PO) involves the removal of ovaries prior to the occurrence of clinically apparent cancer. Benefit of this surgery is an apparent reduction in breast cancer risk by approximately 50% in women with BRCA1/2mutations, presumably due to ablation of ovarian hormones. But may be accompanied by menopausal symptoms and accelerated bone loss [69].

Big Endothelin-1(Big ET-1)

Big ET-1, the biological precursor of endothelin-1(ET-1), a 38amino acid-long peptide that has vasoconstrictive properties [70], after synthesis in the cytoplasm, is cleaved by endothelin converting enzyme to yield active ET-1 (amino acids 1–21) and a C terminal fragment (amino acids 22–38)[71] (Figure 3)[72]. The physiological importance of this conversion of big ET-1into ET-1is that the vasoconstrictive power of the final product is 140 fold greater [73].Big ET-1 has a circulation half-life of 23 minutes compared with only 3.5 minutes for ET-1 [74,75], and was demonstrated to be a more sensitive indicator of endothelin system activation [76-80]. Previous studies have shown elevated plasma big ET-1 levels in patients with various tumors, such as colorectal, non-small cell lung and hepatocellular carcinoma, which were associated with worse outcome [77, 81, 82].

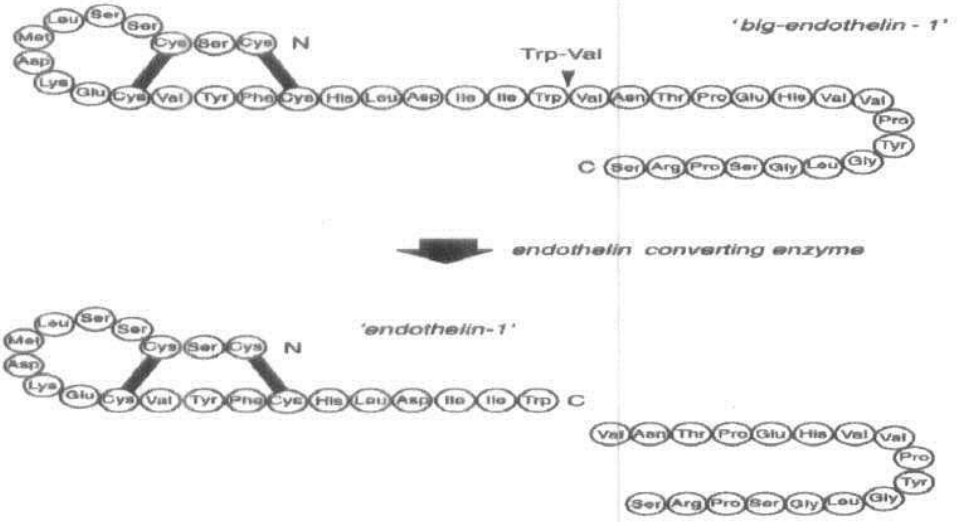


Figure (3): Amino acid sequence of big endothelin-1[72]

Endothelin Synthesis

Endothelins (ETs) are a family of three small peptides ET-1, ET-2 andET-3. ETs mediate their action by interaction with two G-protein coupled receptors (GPCR) subtypes ET_A receptor (ET_AR) and ET_B receptor (ET_BR) [83].

The ET peptides ET-1, ET-2 and ET-3 are encoded by distinct genes, but the three final biologically active products all have 21-amino acids, an α -helical structure, with a hydrophobic C-terminus and two cysteine bridges(disulfide bonds) at the N-terminus. ET-2and ET-3 differs by 2 and 6 amino acids, respectively, from ET-1[84]. Human ET-1 derives from a 212 amino acid precursor, preproendothelin-1, which is intracellularly cleaved by the membrane-bound metalloproteinases endothelin-converting enzyme (ECE-1 and -2) [85]. Further enzymes such furin like endopeptidases, are also thought to be involved. Removal of the signal sequence generates the 195 amino acid proendothelin-1, which is further processed to release the intermediate 38 amino acid 'Big ET-1' [86]. Endothelin-converting enzymes hydrolyze Trp-21-Val-22 bond of big ET-1 to yield the active 21 amino acid ET-1 (Figure 4) [84].

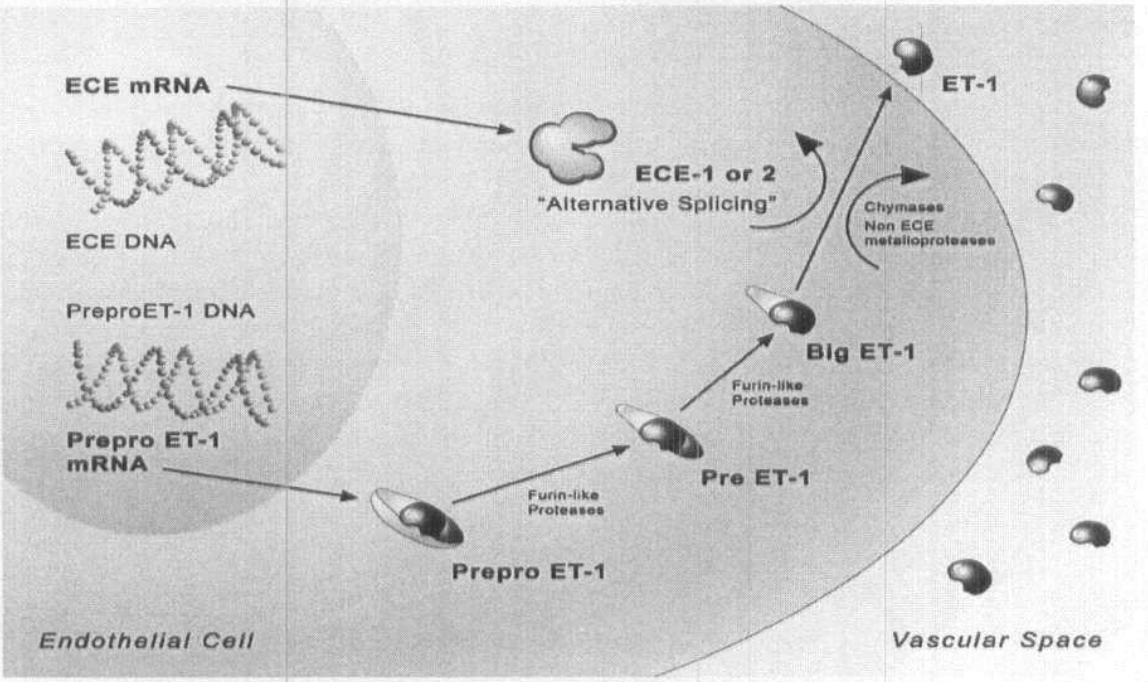


Figure (4): Endothelin-1 biosynthetic pathway [84].

Endothelins in cancer

Increased expression of ET-1 and its receptors have been shown in various human malignancies such as ovarian, prostate [87, 88], and breast cancer [86]. For example, high concentrations of immunoreactive ET-1 have been found in the seminal fluid of patients with advanced prostate cancer [89]. In breast cancers, epithelial and stromal cells also exhibit increased ET-1 immunoreactivity [90].

Direct effects of ET-1 in neoplastic cells appear to primarily involve proliferation and resistance to apoptosis [91], migration and invasion [92]. The mechanism for ET-1 indirect effects may be related to its ability to regulate various kinases, which in turn may have direct effects on cell proliferation, survival, angiogenesis, epithelial to mesenchymal transition, cell motility and invasion, and even cell adherence and metastasis [93-95] (Figure 5) [83]. Further, ET-1 may have indirect effects on tumor metastasis; this appears to occur by stimulation of angiogenesis, in turn resulting from ET-1 stimulation of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) [96]. Additional indirect effects of ET-1 in cancers include on inflammation, extracellular matrix deposition and remodeling [97]. In aggregate, the combination of ET-1 direct cellular effects and indirect effects in the cancer milieu emphasizes the important cell biologic effects of this peptide in carcinogenesis [98, 99].

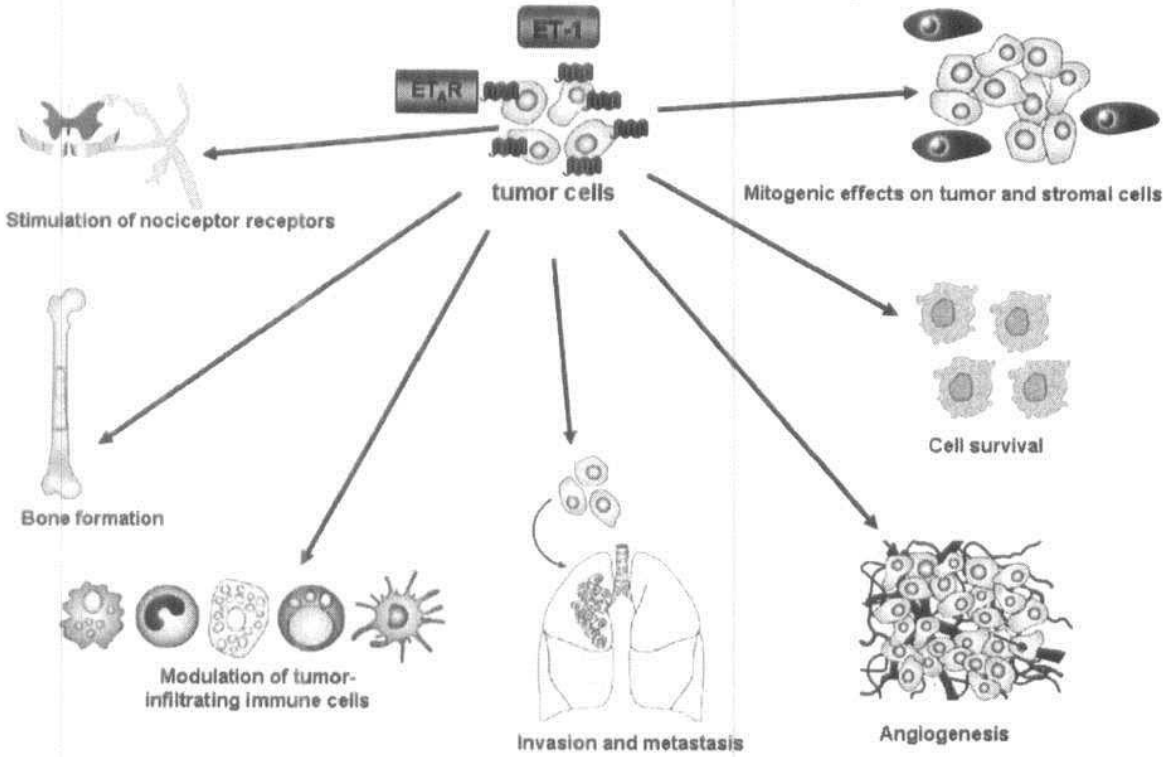


Figure (5): Role of endothelin axis in tumor cells [83].

The endothelin axis in breast cancer

The levels of endothelin synthesis by tumor cells may be stimulated by numerous factors that are present within the breast tumor microenvironment. Solid tumors do not consist of a homogeneous structure or environment: one region of tumor, compared to another, may differ in the levels of hypoxia, cytokine concentration, immune infiltrate, vascularization, necrosis, etc. [100–102]. The breast tumors microenvironment, particularly hypoxia, modulates expression of numerous 'pro-tumor' genes [103], including those of the endothelins and their receptors [97].

Induction of endothelin expression by hypoxia is via the transcription factor hypoxia inducible factor (HIF) 1α [104]. Conversely, endothelins stabilize the HIF1α transcriptional complex leading to expression of angiogenic molecules such as VEGF [105]; accumulation of the HIF1α subunit is associated with breast cancer progression [106]. As well as hypoxia, soluble factors such as cytokines modulate the expression of the endothelin axis in breast tumors. Of particular importance is the finding that endothelins themselves stimulate endothelin receptor production by breast tumor cells [107].

Invasion of breast tumor cells is thought to be promoted by endothelins via several different (autocrine and paracrine) mechanisms including the modulation of matrix metalloproteinase (MMP) activity, induction of proinvasive cytokines and inhibition of anti-invasive cytokines (Figure 6) [86]. Endothelins modulate production of several cytokines by breast tumor cells and tumor associated macrophages (TAMs); ET-1and ET-2 induces expression of the pro-invasive cytokine tumor growth factor-β (TGF-β) by tumor cells via ET_AR whilst inhibiting expression of the anti-metastatic cytokine interleukin-10 (IL-10) by macrophages [107].

ET-1/ET_AR interactions affect key players in metastasis, such as MMPs and the urokinase type plasminogen activator system [108]. ET-1 stimulates lymphatic vessels and lymphatic endothelial cells to grow and invade [109].

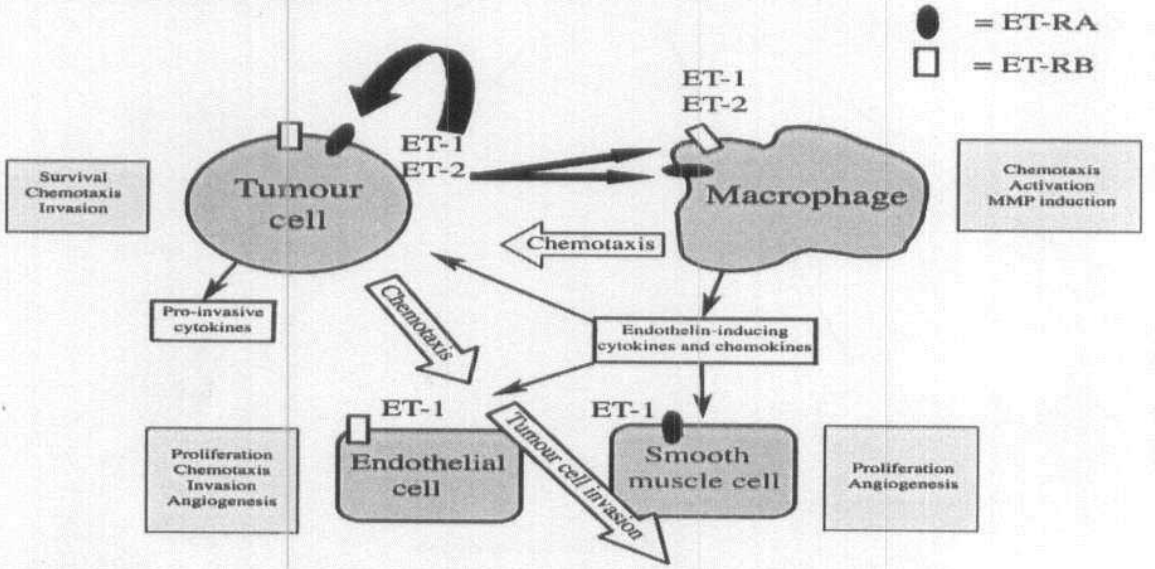


Figure (6): Putative roles of endothelins in breast tumor cell invasion [86]. Tumor infiltrating macrophages express inflammatory cytokines that may induce endothelin expression by both tumor cells and macrophages, which release ET-1 and ET-2, and express both receptors. Micro environmental factors, including hypoxia and the endothelins themselves, further stimulate the endothelin axis. Stimulation of tumor cells and macrophages with ET-1 or ET-2 leads to chemotaxis of these cells, induction of MMP activity and cytokine expression, and invasion of the tumor cells. ET-2 also protects tumor cells from apoptosis and activates macrophages. Endothelial cells and vascular smooth muscle cells (VSMC) express ET-RB and ET-RA, respectively. Stimulation of endothelial cells and VSMC with endothelins may stimulate angiogenesis.

Tumor Markers

Tumor markers are biochemical substances elaborated by tumor cells. These markers can be normal endogenous products that are produced at a greater level in cancer cells or the products of newly switched on genes that remained quiescent in the normal cells [110]. Biochemical markers can be useful for screening, diagnosis, prognosis, monitoring, staging and overall management of cancers such as, breast, pancreas, ovary, germ cell tumors and colorectal cancer [111, 112].

There is tremendous variety of biomarkers, which can include proteins (e.g., an enzyme or receptor), nucleic acids (e.g., microRNA or other non-coding RNA), antibodies, and peptides, among other categories. It can be detected in the circulation (whole blood, serum, or plasma) or excretions or secretions (stool, urine, sputum, or nipple discharge), and thus easily assessed non-invasively and serially, or can be tissue-derived, and require either biopsy or special imaging for evaluation [112].

Tumor markers are usually not used alone for the diagnosis because most markers can be found in elevated levels in people who have benign conditions, and because no tumor marker is yet specific to a particular cancer.Prognostic markers that are utilized extensively by clinicians can be correlated with an endpoint regardless of therapy. On the other hand, predictive biological indicators predict outcome to a specific therapy (Figure 7)[113-115].

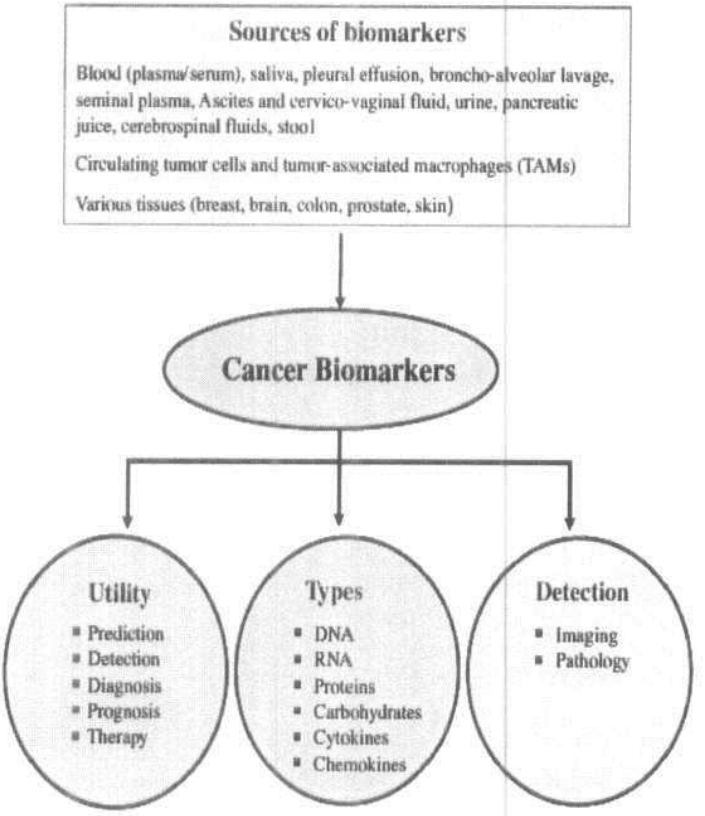


Figure (7): Overview of cancer biomarkers: sources, types and potential applications[115].

Tumor Markers in Breast Cancer

Several tumor markers, both tissue-based and serum based are currently used in the management of patients with breast cancer [112,116-118].

Tumor markers have been of great use in characterizing breast tumors; in this sense, a multitude of molecules involved in breast cancer biology have been studied as potential prognostic markers. Well-identified molecular markers such as the proliferation marker (Ki-67), hormone receptors (estrogen and progesterone receptors), and Her-2 are routinely used to make treatment decisions in patients with early stage breast cancer. Other tumor markers that have demonstrated prognostic value are not routinely used, including cyclin D1, cyclin E, p53 nuclear protein accumulation, and Bcl-2 (B-cell lymphoma2) expression. There are also other molecular markers for which there is not enough evidence for them to be used as prognostic markers [119, 120].

ER and PR

The first and still one of the best therapy predictive markers in oncology is the estrogen receptor (ER), which is used in selecting patients with breast cancer for endocrine hormone therapy [121]. In an attempt to improve the positive predictive accuracy of ER; several additional markers have been investigated. The most widely used of these is the progesterone receptor (PR). The original hypothesis for investigating PR as a marker of endocrine sensitivity in patients with breast cancer was based on the finding that this protein was induced by estrogen acting via the ER. PR was thus hypothesized to be a marker for a functional or active ER [122].

Human Epidermal Growth Factor Receptor 2 (HER-2)

The HER-2 gene product is a transmembrane protein normally involved in cell growth and differentiation through its interaction with circulating growth factors. Identified as an oncogene, amplification results in protein overexpression that supports rapid cellular proliferation. Seen in approximately 25% of breast cancer patients, overexpression (HER-2-positive tumors) is associated with a more aggressive clinical course and worse overall prognosis [123]. HER-2 status has also been shown to be somewhat predictive of responsiveness to various chemotherapeutic agents [124].

Carbohydrate antigen 15.3(CA15.3)

CA 15.3, also known as MUC1, episialin, polymorphic epithelial mucin or epithelial membrane antigen, is the most widely used marker in breast cancer. It is a large transmembrane glycoprotein containing three main domains: a large extracellular region, a membrane-spanning sequence and a cytoplasmic domain [125].

Structure and function of MUC-1

The human mucin (MUC) family consists of members designated MUC1 to MUC21 that have been sub-classified into secreted and transmembrane forms. The secreted mucins (for example, MUC2, MUC5AC, MUC5B and MUC6) form a physical barrier, which as a mucous gel provides protection for epithelial cells that line the respiratory and gastrointestinal tracts and form the ductal surfaces of organs such as the liver, breast,

pancreas and kidney. The transmembrane mucins (for example, MUC1, MUC4, MUC13 and MUC16) have a single membrane-spanning region and contribute to the protective mucous gel through their ectodomains of *O*-glycosylated tandem repeats that form rod-like structures that extend over 100 nm from the cell surface[126].

In tissue, MUC-1 exists as a transmembrane protein consisting of 2 subunits that form a stable dimer [126, 127]. The MUC-1 amino terminal subunit contains variable numbers of tandem repeats with a high proportion of prolines, threonines and serine (which constitute the PTS domain) that are modified by glycosylation. The number of tandem repeats varies between approximately 25 and 100 in different individuals. This subunit which extends 200–500 nm beyond the cell membrane has been implicated in cell adhesion/deadhesion. The extracellular domain of MUC-1 can be released into the extracellular matrix and thus shed into serum. The release has been shown to be mediated by 2 proteases, a disintegrin and metalloproteinase17 (ADAM17) [128] and membrane type-matrix metalloproteinase1 (MT-MMP1) [129]. The amino terminal subunit is attached to the membrane via non-covalent binding to the carboxy terminal subunit. The carboxy terminal subunit consists of a 58-amino acid extracellular domain, a 28-amino acid transmembrane domain and a 72-amino acid cytoplasmic domain. Like the amino terminal, the carboxy terminal subunit can also undergo glycosylation. The carboxy terminal peptide has been shown to play a role in cell signaling. This signaling appears to be mediated following its interaction with a number of kinases including c-src, epidermal growth factor (EGFR), protein kinase c delta (PKCδ), phosphatidyl inositol-3 kinase (PI3K), and non-kinase proteins such as β-catenin, growth factor receptor bound protein-2 (GRB-2) and estrogen receptor (Figure 8) [126,130].

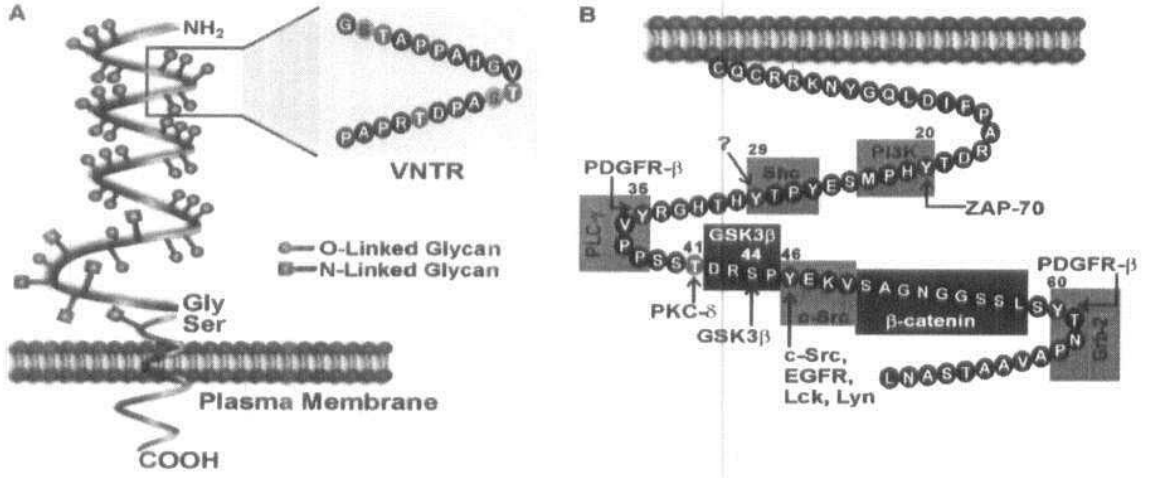


Figure (8): Structure of MUC1 [130]. (A) The extracellular region contains sites of O- and N-linked glycosylation. Serines and threonines are the sites of O glycosylation, while asparagines are potential sites of N-glycosylation. The 20–amino acid variable number of tandem repeats (VNTR) is also referred to as the PTS domain due to the preponderance of proline, threonine, and serine residues (colored ovals). Although only 6 VNTRs are shown, their actual number can vary from 25 to 125 in various tissues and/or individuals. Proteolysis at the indicated Gly-Ser site creates a heterodimer protein structure. (B) Tyrosine residues (purple) in the MUC1 are phosphorylated by the indicated kinases (arrows) and serve as binding sites (boxes) for kinases and adapter proteins. Additional binding sites for glycogen synthase kinase 3β (GSK3β) and β-catenin are indicated. The question mark indicates the action of an unknown kinase.

MUC-1 in cancer formation and progression

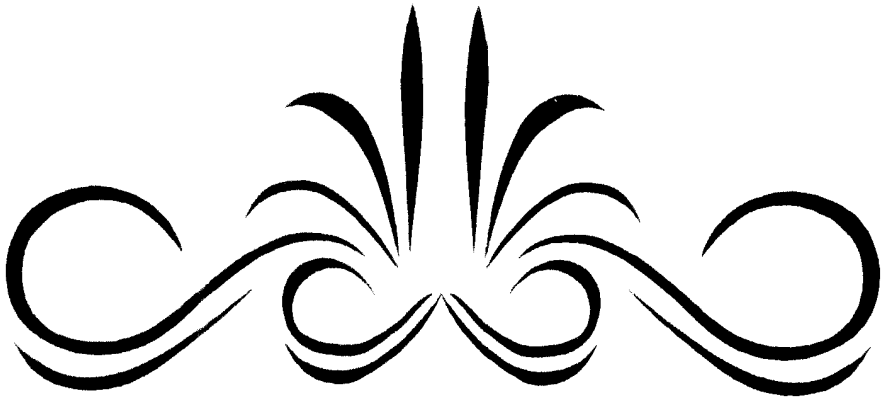
In normal secretory epithelial cells, MUC-1 is expressed at the apical plasma membrane. However, following malignant transformation, MUC-1 may be expressed at high levels on the entire membrane surface as well as in the cytoplasm. In addition to the altered cellular localization, changes in glycosylation may occur during malignant transformation as a result of changes in glycosyltransferase expression in malignant cells[126, 127]. Although the numbers of glycosidic links are increased, the carbohydrate chains are generally shorter [131]. Thus, overexpression and altered glycosylation allows MUC-1 to be used a cancer marker. The clinical utility of MUC-1 is confined to the measurement of its shed or soluble forms in serum, by assays such as CA 15.3 [132, 133]. Some of these soluble forms result from proteolytic shedding of the extracellular domain of MUC-1. However, some may also be derived from alternative splicing of MUC-1 mRNA, resulting in products lacking the transmembrane domain [127].

Altered expression of MUC-1 however, has been implicated in the pathogenesis of cancer formation and metastasis. Both the amino and carboxy terminal subunits appear to contribute to oncogenic activity [127, 134]. The carboxy terminal subunit appears to drive cancer formation by interacting with the signalling proteins mentioned, thus driving cell proliferation. MUC1 interacts with β-catenin which is an oncogenic protein that contributes to the metastasis of several types of malignant epithelial cells, results in its redistribution to the margin of invading cells, in turn leading to increased invasiveness of the tumor cells[135]. On the other hand, the amino terminal subunit may play a role in cancer formation/progression via its interaction with adhesion proteins such as Intercellular adhesion molecule 1 (ICAM-1) present on endothelial cells [136]. Interaction of MUC-1 with ICAM-1 has been shown to suggesting a role in invasion and/or metastasis. The extended N-terminal domain of MUC-1 has also been implicated in blocking access of immune cells to tumors [127, 137]. Because of its role in tumor formation and progression, MUC-1 has been referred to as an oncogene [138, 139].

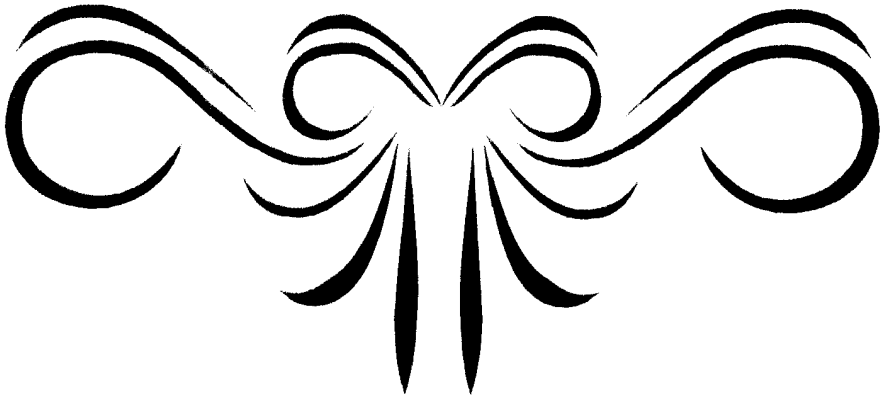
CA15.3 in cancer diagnosis and prognosis

Numerous studies have confirmed that CA15.3 is among the best available serum markers for breast cancer [112]. However CA15.3 uses for screening and diagnosis is limited by low sensitivity in early-stage disease and lack of specificity [132]. As a result, CA15.3 is not recommended by any oncology group for screening, diagnosis, or staging of breast cancer [112]. CA15.3 has achieved Food and Drug Administration (FDA) clearance for monitoring recurrence in breast cancer patients with advanced disease [112].

Although elevated levels of CA15.3 are found in the majority of breast cancer patients with distant metastasis, high concentration may also occur in patients with several different types of advanced adenocarcinoma. Advanced adenocarcinoma, other than breast, that may give rise to elevated levels of CA15.3 include ovarian, pancreatic, gastric and lung cancer [140-142]. According to the European Group on Tumor Markers (EGTM) guidelines CA15.3 should be used for monitoring therapy in patients with advanced breast cancer [143]. According to this Panel, "marker levels should be measured prior to every chemotherapy course and at three monthly intervals for patients receiving hormone therapy" [143]. The American Society of Clinical Oncology (ASCO) guidelines stating that in monitoring therapy in metastatic breast cancer, CA15.3 should not be used alone but can be used in conjunction with diagnostic imaging, history and physical examination [144,145].



Aim of The Work



AIM OF THE WORK

The purpose of this study was to investigate serum big ET-1 levels in patients with breast cancer and its correlation with CA15.3, clinical and pathological criteria as well as outcome of the disease.


Patients
And
Method

PATIENTS AND METHODS

Patients:

The present study was carried out on 40 females with newly diagnosed breast cancer (age range, 35-80 years; median age, 49years) selected from those admitted to Surgical Department and then they are followed in the Department of Cancer Management and Research at Medical Research Institute, Alexandria University from 2009 to 2011 and 15 healthy females were considered as a control group (age range, 27 -75 years; median age, 49 years). Breast cancer females were divided into the following groups:-

- Non metastatic group: included 31 female patients with primary breast cancer of different clinical stages but without any metastasis.
- Metastatic group: included 9 patients with metastatic breast cancer who had undergone treatment for their diseases and in whom metastasis was detected during follow-up.

All patients met the following criteria:

- Having primary invasive breast carcinoma.
- No clinical manifestation of infection, autoimmune disease or immune deficiency disease and cardiac disease.
- Not receiving immunomodulatory agent in recent 3 weeks.
- Not receiving blood transfusion in recent 3 weeks.

All patients were subjected preoperatively to the following:

- Full medical history taking.
- Thorough clinical examination.
- Mammography of breast and ultrasonography of abdomen.
- Laboratory investigations including: Complete blood picture, liver and kidney functions, fasting blood sugar, and bleeding and coagulation times.
- Fine needle aspiration and/or core cutting needle biopsy to detect malignancy.
- Radiological investigations including: Chest X-ray, liver ultrasound, isotopic bone scan for detection of metastasis even postoperatively.

All breast cancer patients were treated by different surgical techniques followed by proper adjuvant treatment protocols based on tumor stage and risk factors.

This study was approved by the local ethical committees of Medical Research Institute, Alexandria University. Also assigned informed consent was obtained from all the participants in the present study.

Methods:

Blood sampling:

Five ml blood samples were withdrawals from all patients one day before surgery, after both surgery and after finishing the appropriate adjuvant treatment protocols immediately and from control subjects. All blood samples were collected in specimen tubes, left to clotted then centrifuged at 25°c for 10 minutes; serum supernatant was stored at -80 °c until used for determination of big endothelin-1(Big ET-1) and CA15.3.

1- Big Endothelin-1 (Big ET-1) Assay [146]

Levels of big endothelin-1 were measured with an enzyme immunoassay (EIA) kit (Biomedica Group GmbH, Vienna, Austria).

Principle of the method:

The kit uses a polyclonal sheep anti big endothelin-1 antibody immobilized on a micro titer plate to bind the big ET-1 in the standards or sample. A recombinant human big ET-1 standard was provided in the kit. After addition of sample or standard a monoclonal antibody to big ET-1 labeled with the enzyme horseradish peroxidase was added. This labeled antibody binds to the big ET-1 captured on the plate. After a short incubation the excess labeled antibody was washed out and substrate was added. The substrate reacts with the labeled antibody bound to the big ET-1 on the plate. After a short incubation, the enzyme reaction was stopped and the color generated was read at 450 nm. The measured optical density was directly proportional to the concentration of big ET-1 in either standards or samples.(Figure 9)

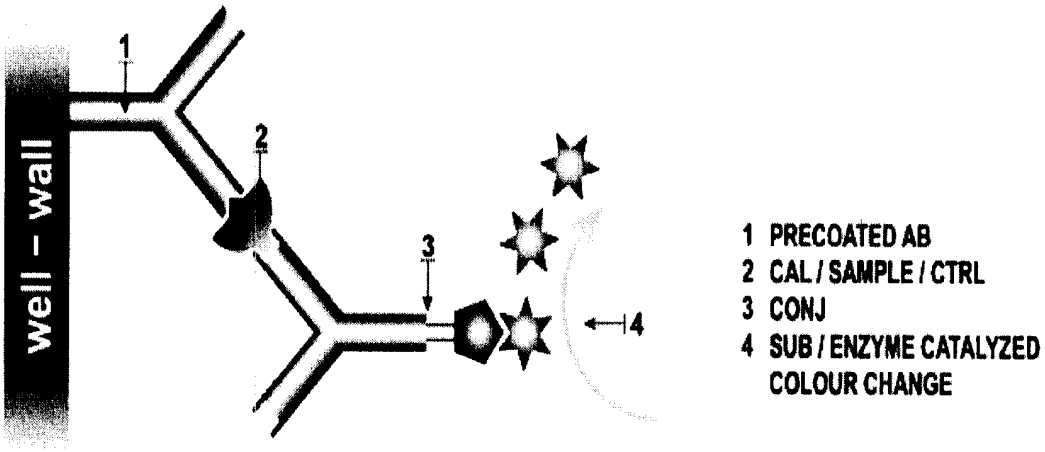


Figure (9): Principle of enzyme immunoassay

Reagents:

1) Polyclonal sheep anti big endothelin-1antibody coated microtiter plate.
2) Wash buffer concentrate.
3) Human big endothelin-1 standard, lyophilized. (S1 to S5 are 9, 3, 1, 0.33, 0 Fmol/ml)
4) Human big endothelin-1 control, lyophilized.
5) Conjugate, monoclonal anti big endothelin-1 antibody horseradish peroxidase (HRP) labeled, ready to use.
6) Substrate 3, 3', 5, 5' - tetramethylbenzidine (TMB solution), ready to use.
7) Stop solution, ready to use.

Reagents preparation:

- STD (Standards) 1 to 5 bottle was reconstituted in 0.5 ml distilled water at room temperature (18-26°c) for 20 minute, shake well.
- CTRL (Controls) was reconstituted in 0.5ml distilled water at room temperature (18-26°c for 20 minute, shake well.
- Wash buff (Wash buffer) concentrate was diluted 1:20 with distilled water. Crystals in the buffer concentrate was dissolved at room temperature.

Assay:

All reagents and samples were brought to room temperature (18-26°c) before performing the assay.

1) 50 µl of standards, control and serum were added into respective wells, except blank.
2) 200 µl of conjugate was added into each well, except blank, swirl gently.
3) Plate was cover tightly and incubated 4 hours at room temperature (18-26°c) in the dark.
4) Wells were aspirated and washed 5 times with 300µl diluted wash buffer; remaining wash buffer was removed by hitting plate against paper towel after the last wash.
5) 200 µl of substrate (TMB) was added into each well. Plate was incubated for 30 minutes at room temperature in the dark.
6) 50 µl of stop solution were added into each well, shake well.
7) Absorbance was measured immediately at 450 nm.

Calculation of results:

1) Subtract the blank extinction from all other values.
2) A standard curve was constructed by plotting the absorbance value of each standard (y-axis) against corresponding concentration (x-axis). (Figure 10).
3) Concentration of samples was obtained from standard curve.

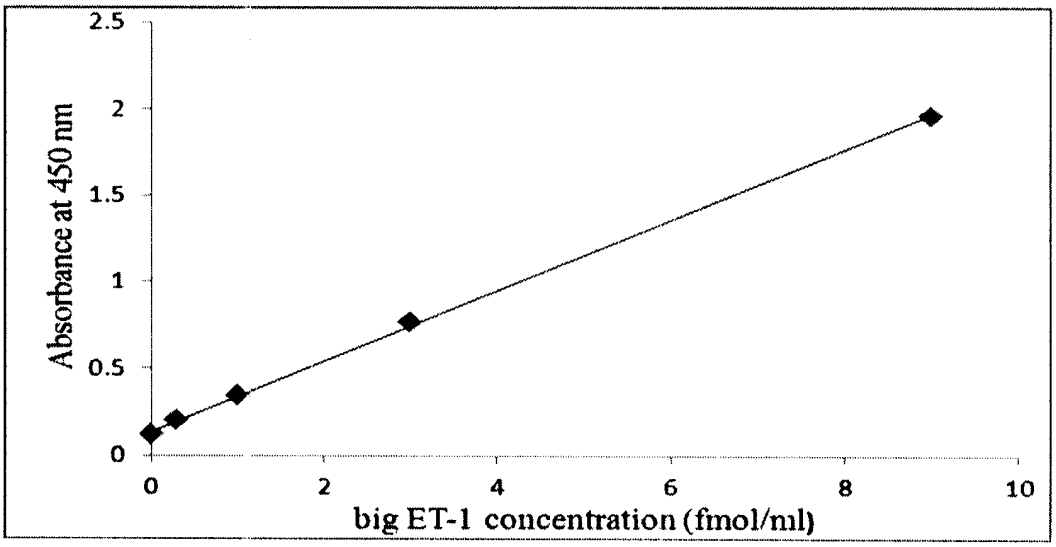


Figure (10): Big endothelin-1 standard curve

2-Carbohydrate Antigen15.3 (CA15.3) Assay [147]

Levels of CA15.3 were measured with immunoradiometric assay (IRMA) (DIAsource Immuno Assay S.A. Belgium).

Principle of the method:

Immunoradiometric assay based on coated-tube separation. The capture antibody was 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 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. (Figure 11)

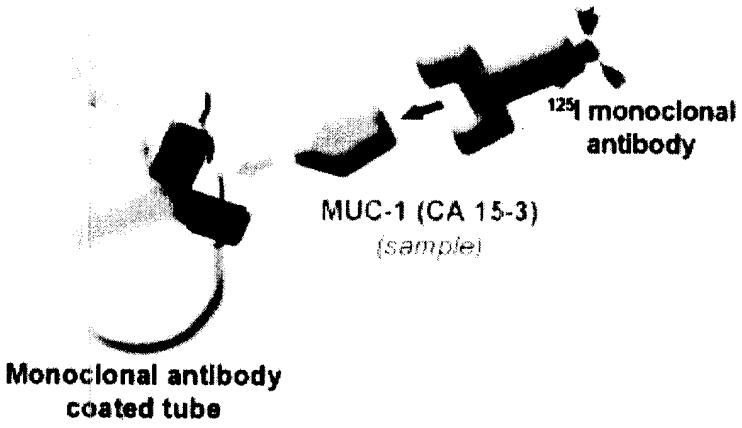


Figure (11): Principle of immunoradiometric assay

Reagents:

1) Tubes coated with anti CA15.3 (monoclonal antibody), ready for use.
2) Anti- CA15.3- ^{125}I (monoclonal antibody) in phosphate buffer with bovine serum, azide (<0.1%) and inert red day, ready for use.
3) Calibrators 1-5 in phosphate buffer with bovine serum albumin and thymol (<0.1%), lyophilized. (S1 to S5 are 318, 99, 22.4, 9, 2.7, 0 u/ml).
4) Dilution buffer: phosphate buffer with bovine serum and azide (<0.1%), ready for use.
5) Wash solution (TRIS-HCL).
6) Controls in human serum and thymol, lyophilized.

Reagents preparation:

- Calibrators: calibrators were reconstituted with 0.5ml dilution buffer.
- Controls: controls were reconstituted with 0.5 ml distilled water.
- Working wash solution: an adequate volume of working solution was prepared by adding 69 volumes of distilled water to 1 volume of wash solution (×70). Magnetic stirrer was used to homogenize.

Assay:

All reagents and samples were brought to room temperature prior to be used; all reagents and sample were thoroughly mixed by gentle agitation or swirling.

1) Plain of plastic tubes were labeled for each sample and control.
2) 250µl of dilution buffer was dispensed into each tube.
3) 10µl of samples and controls were added into these tubes.
4) Coated tubes were label for each calibrator, sample and control.
5) Calibrators, pre-diluted samples and controls were briefly vortex and dispensed 50µl of each into the respective tubes.
6) The tubes were incubated for 90 minutes at room temperature on tube shaker (400rpm).
7) The contents of each tube (except total count) were aspirated. Be sure that the plastic tip of the aspirator reaches the bottom of the coated tubes in order to remove all the liquid.
8) The tubes were washed 2 times with 2 ml working solution (except total count) and aspirated. Foaming during addition of working solution was avoided.
9) The tubes let to stand upright for 2 minutes and the remaining drop of liquid was aspirated.
10) 50µl of ^{125}I labeled anti CA15.3 was dispensed into each tube, including the uncoated tubes for total count.

11) Tubes were incubated for 90 minutes at room temperature on tube shaker (400rpm).

12) The contents of each tube were aspirated (except total counts). Be sure that the plastic tip of the aspirator reaches the bottom of the coated tubes in order to remove all the liquid.

13) Tubes were washed 2 times with 2 ml working solution (except total count) and aspirated. Foaming during addition of working solution was avoided.

14) The tubes let to stand upright for 2 minutes and the remaining drop of liquid was aspirated.

15) Tubes were counted in a gamma counter for 60 seconds.

Calculation of results:

1) On semi logarithmic or linear graph paper the c.p.m for each calibrator was plotted against the corresponding concentration of CA15.3 and calibration curve was drawn through the calibrator points. (Figure 12)

2) The concentration for each control and sample were read by interpolation on calibration curve.

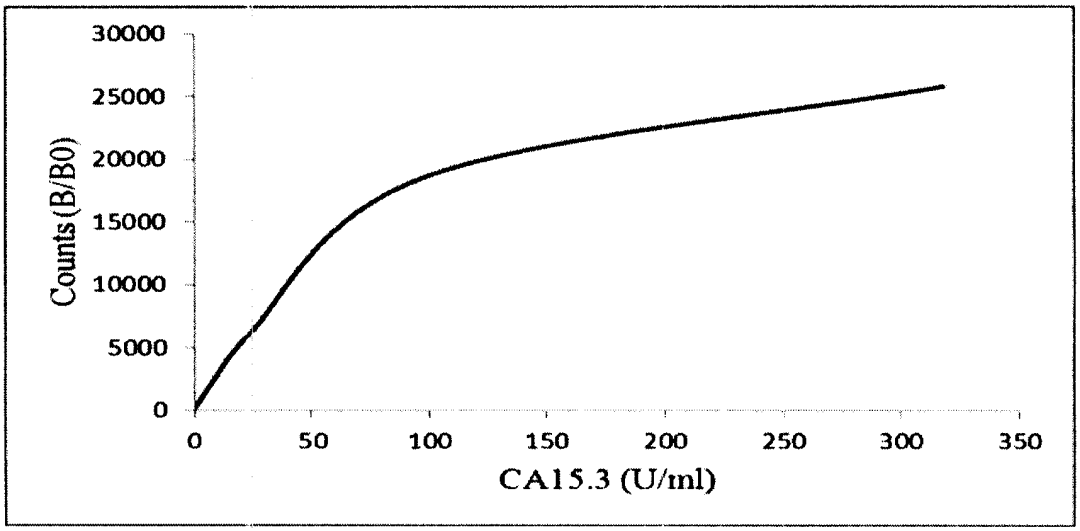


Figure (12): CA15.3 standard curve

Statistical analysis of the data:

Data were fed to the computer using the Predictive Analytics Software (PASW Statistics 18).Quantitative data were described using minimum and maximum as well as mean and standard deviation.

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. If it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used.

For abnormally distributed data, Mann-Whitney Test (for data distribution that was significantly deviated from normal) was used to analyze two independent populations. Wilcoxon signed ranks test was used to compare between the different periods. A difference was considered significant at ($p \leq 0.05$). Correlations between two quantitative variables were assessed using Spearman coefficient.

Agreement of the different predictives with the outcome was used and was expressed in sensitivity (which defined as proportion of people with disease who will have a positive result {a/ (a + c)}) and specificity (which defined as the proportion of people without the disease who will have a negative result {d/ (b + d)}), where: a = true positive cases, c = false negative cases, d = true negative cases and, b = false positive cases [148]. Receiver operating characteristic curve (ROC) was plotted to analyze a recommended cutoff (which is the upper normal limits), the area under the ROC curve denotes the diagnostic performance of the test. Area more than 50% gives acceptable performance and area about 100% is the best performance for the test.


Results

RESULTS

The results obtained in the present study were illustrated as follows:

• Clinicopathological parameters and characteristics of breast cancer patients group:

The general characterizations of all breast cancer patients (metastatic and non metastatic) are represented in Table (6). The Table showed that 2 patients (5%) had T1 tumor (<2 cm), 33 patients (82.5%) had T2 tumor (2-5 cm) and 5 patients (12.5%) had T3 tumor (>5 cm). Negative lymph nodes were observed in 30% of cases whereas 70% of patients were positive. With respect to pathological stage the table showed that 2.5% of patients in pathological stage I, 62.5% of stage II, 12.5% of stage III and 22.5% of stage IV. The table showed that all of cases were of histological grade II (77.5%) and III(22.5%). With respect to pathology type 92.5% of patients appeared to have invasive ductal carcinoma while 7.5% of patients have invasive lobular carcinoma. Most of patients were estrogen receptor (ER) and progestrone receptor (PR) positive. 35% of patients with human epidermal growth factor-2 (HER-2) positive. Vascular invasion was presented in (47.5%) of the cases, while metastasis was detected during follow up period in 9 patients (22.5%) in which, 3 patients were developed metastasis in bone, metastasis were occured in uterus in 2 patients, 2 other patients metastasis were appeared in ovary, only 1 patient was developed metastasis in brain and 1 other patient was developed metastasis in liver. According to menopausal status 55% of patients premenopause while, 45% of patients postmenopause. With respect to surgical treatment 95% of patients were undergo to mastectomy but only 5% of patients undergo conservative surgery. Accorging to adjuvant treatment 50% of patients take adjuvant chemotherapy, 10% take hormonal therapy, 17.5% take both chemotherapy and radiotherapy, 10% take chemotherapy and hormonal therapy and 12.5% take chemotherapy, radiotherapy and hormonal therapy.

Table (6): Clinicopathological parameters and characteristics of breast cancer patients group

Clinicopathological parameter and patient characteristics		Patient group (n=40)	Percent (%)
Tumor size	<2 (T1)	2.0	5.0
	2-5 (T2)	33.0	82.5
	>5 (T3)	5.0	12.5
Lymph Node status(LN)	-ve	12.0	30.0
	+ve (1-3)	15.0	37.5
	+ve (4-9)	11.0	27.5
	+ve ≥10	2.0	5.0
Pathological stage	I	1.0	2.5
	II	25.0	62.5
	III	5.0	12.5
	IV	9.0	22.5
Histological grade	II	31.0	77.5
	III	9.0	22.5
Pathology type	Invasive ductal carcinoma	37.0	92.5
	Invasive lobular carcinoma	3.0	7.5
ER status	-ve	2.0	5.0
	+ve	34.0	85.0
	Unknown	4.0	10.0
PR status	-ve	3.0	7.5
	+ve	33.0	82.5
	Unknown	4.0	10.0
HER-2 status	-ve	5.0	12.5
	+ve	14.0	35.0
	Unknown	21.0	52.5
Vascular invasion	-ve	21.0	52.5
	+ve	19.0	47.5
Metastasis	-ve	31.0	77.5
	+ve	9.0	22.5
Menopausal status	Premenopause	22.0	55.0
	Postmenopause	18.0	45.0
Treatment			
Surgical	Mastectomy	38.0	95.0
	Conservative surgery	2.0	5.0
Adjuvant	Chemotherapy	20.0	50.0
	Hormonal therapy	4.0	10.0
	Chemo + radiotherapy	7.0	17.5
	Chemo + hormonal therapy	4.0	10.0
	Chemo + radio+ hormonal therapy	5.0	12.5

• Biochemical results

Serum big endothelin-1(big ET-1)(fmol/ml)

Individual data, range, mean ± S.D and SE values of big ET-1 in normal control group and breast cancer patients group, before surgery and after surgery and treatment were showed in Table (7). Statistical analyses of these results were represented in Table (8) and illustrated in Figure (13).

As presented in Table (7), serum big ET-1 (fmol/ml) was ranged from 0.30 - 0.89 with a mean value of 0.62 ± 0.17 in control group, from 1.04 - 8.2 in breast cancer patients without metastatic before surgery with a mean 2.94 ± 1.42, from 0.37 - 1.02 with a mean value of 0.63 ± 0.14 in those patients after surgery and treatment, from 1.11 - 3.85 in breast cancer patients with metastatic before surgery with a mean 2.97 ± 0.92 and from 3.33 - 5.41 with a mean value of 4.05 ± 0.70 in those patients after surgery and treatment.

The statistical analyses of these results showed that the serum big ET-1 of breast cancer patients either with or without metastatic before surgery was significantly higher than those of control group ($p<0.001$). Mean value of serum big ET-1 of breast cancer patients without metastatic after surgery and treatment showed insignificant difference as compared with control group ($p=0.677$).While, it was significantly lower as compared with their corresponding values before surgery and become similar to the normal control level ($p=<0.001$). On the other hand, serum big ET-1 level of metastatic patients after surgery and treatment was significantly higher than control group, than their corresponding values before surgery and than breast cancer patients without metastatic after surgery and treatment ($p_1=<0.001$, $p_2= 0.02$ and $p_3= <0.001$ respectively).

Table (7): Serum big ET-1 (fmol/l) level of control group and breast cancer patients groups before surgery and after surgery and treatment

Number	Control group (n=15)	Breast cancer patients groups without metastatic (n=31)		Breast cancer patients groups with metastatic (n=9)	
		Before surgery	After surgery and treatment	Before surgery	After surgery and treatment
1	0.68	2.85	0.39	3.81	3.33
2	0.39	2.72	0.64	2.4٠	3.64
3	0.35	4.49	0.52	2.68	5.41
4	0.89	3.9٠	1.02	3.85	4.22
5	0.66	3.02	0.59	2.36	4.12
6	0.68	3.1٠	0.66	1.11	3.33
7	0.65	2.93	0.64	3.81	4.56
8	0.66	1.87	0.46	3.39	3.43
9	0.72	2.65	0.49	3.31	4.42
10	0.85	2.77	0.69		
11	0.3٠	3.4٠	0.79		
12	0.59	5.26	0.72		
13	0.68	2.65	0.6٠		
14	0.74	1.04	0.48		
15	0.55	1.44	0.52		
16		1.4٠	0.69		
17		2.52	0.77		
18		2.31	0.93		
19		2.24	0.69		
20		3.02	0.61		
21		2.28	0.63		
22		2.35	0.58		
23		2.72	0.37		
24		1.94	0.48		
25		8.2٠	0.7٠		
26		5.86	0.61		
27		1.85	0.75		
28		2.56	0.53		
29		2.69	0.6٠		
30		3.43	0.62		
31		1.76	0.67		
Range	0.30 - 0.89	1.04 - 8.2	0.37- 1.02	1.11 – 3.85	3.33 - 5.41
Mean ± SD	0.62 ± 0.17	2.94 ± 1.42	0.63 ± 0.14	2.97 ± 0.92	4.05 ± 0.70
SE	0.04	0.26	0.03	0.31	0.23

Table (8): The statistical analyses of serum big ET-1 levels (fmol/ml) in control group and breast cancer patients groups before surgery and after surgery and treatment

Big ET-1	Control group (n=15)	Breast cancer patients groups without metastatic (n=31)		Breast cancer patients groups with metastatic (n=9)	
		Before surgery	After surgery and treatment	Before surgery	After surgery and treatment
Mean ± SD	0.62 ± 0.17	2.94 ± 1.42	0.63 ± 0.14	2.97 ± 0.92	4.05 ± 0.70
P1		<0.001*	0.677	<0.001*	<0.001*
P2			<0.001♦		0.02♦
P3					<0.001¤

Differences were considered statistically significant at $p \leq 0.05$.

P1 (*): values as compared to control group using Mann Whitney test.

P2 (♦): values as compared to breast cancer patients group before surgery using Wilcoxon signed ranks test.

P3 (¤): values as compared to breast cancer patients group without metastatic after surgery and treatment using Mann Whitney test.

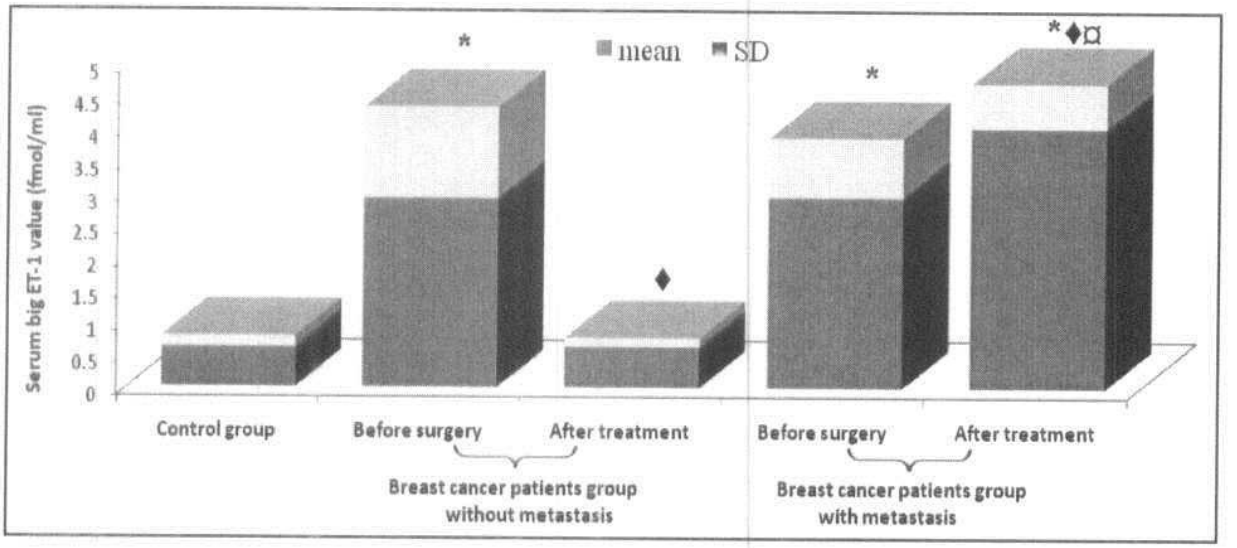


Figure (13): Serum big ET-1 (fmol/ml) in control group and breast cancer patients groups before surgery and after surgery and treatment

* : significantly different from control group.

♦ : significantly different from breast cancer patients group before surgery.

¤ : significantly different from breast cancer patients group without metastatic after surgery and treatment.

Serum cancer antigen 15.3 (CA15.3) (U/ml)

Individual data, range, mean ± S.D and SE values of CA15.3 in control group and breast cancer patients group, before surgery and after surgery and treatment are shown in Table (9). Statistical analyses of these results were represented in Table (10) and illustrated in Figure (14).

As presented in Table (9), in control group, serum CA15.3(U/ml) was ranged from 7.8 - 22.3 with a mean value of 13.14 ± 4.21, in breast cancer patients without metastatic before surgery from 5.9 - 54.6 with a mean 18.38 ± 11.16, in those patients after surgery and treatment from 5.4 - 24.7 with a mean value of 15.95 ± 4.91, in breast cancer patients with metastatic before surgery from 9.50 - 34.15 with a mean 19.07 ± 9.23 and in those patients after surgery and treatment from 69.90 - 134.00 with a mean value of 89.32 ± 19.81.

The statistical analyses of these results showed that the level of serum CA15.3 in non metastatic patients either before surgery or after treatment showed insignificantly difference as compared to either control group or to each other (p= 0.094, 0.061 and 0.563 respectively).

In metastatic patients, CA15.3 values before surgery showed insignificantly difference as compared to control group (p=0.128). while after surgery and treatment, it was significantly higher than control group, than their corresponding values before surgery and than breast cancer patients without metastatic after surgery and treatment (p_1=<0.001, p_2 = 0.008 and p_3= <0.001 respectively).

Table (9): Serum CA15.3 (U/ml) level of control group and breast cancer patients groups before surgery and after surgery and treatment

Number	Control group (n=15)	Breast cancer patients groups without metastatic (n=31)		Breast cancer patients groups with metastatic (n=9)	
		Before surgery	After surgery and treatment	Before surgery	After surgery and treatment
1	12.50	21.0	22.20	30.60	75.50
2	12.00	40.30	24.70	11.00	88.90
3	15.20	11.10	13.80	34.15	82.60
4	16.70	17.94	16.23	11.30	73.20
5	17.50	15.30	18.40	10.40	69.90
6	8.10	25.70	14.50	9.50	85.20
7	7.80	12.60	18.60	23.69	105.24
8	8.90	7.20	7.90	19.07	89.32
9	11.10	12.40	17.80	21.90	134.00
10	9.30	25.70	15.95		
11	10.30	19.50	16.10		
12	12.60	19.50	11.90		
13	14.60	23.80	19.20		
14	18.20	6.20	5.40		
15	22.30	6.60	11.00		
16		8.40	10.90		
17		16.30	13.50		
18		24.90	14.40		
19		14.80	23.60		
20		15.00	20.50		
21		12.90	18.10		
22		17.40	22.30		
23		17.60	21.80		
24		18.38	6.50		
25		٤٥.٦٥	21.70		
26		14.30	14.40		
27		5.90	10.70		
28		18.50	13.00		
29		54.60	15.70		
30		11.90	16.00		
31		8.40	17.70		
Range	7.8 - 22.3	5.9 - 54.6	5.4 - 24.7	9.50 - 34.15	69.90 - 134.00
Mean ± SD	13.14 ± 4.21	18.38 ± 11.16	15.95 ± 4.91	19.07 ± 9.23	89.32 ± 19.81
SE	1.09	2.01	0.88	3.08	6.6

Table (10): The statistical analyses of serum CA15.3 (U/ml) in control group and breast cancer patients groups before surgery and after surgery and treatment

CA15.3	Control group (n=15)	Breast cancer patients groups without metastatic (n=31)		Breast cancer patients groups with metastatic (n=9)	
		Before surgery	After surgery and treatment	Before surgery	After surgery and treatment
Mean ± SD	13.14 ± 4.21	18.38 ± 11.16	15.95 ± 4.91	19.07 ± 9.23	89.32 ± 19.81
P1		0.094	0.061	0.128	<0.001*
P2			0.563		0.008♦
P3					<0.001¤

Differences were considered statistically significant at $p \leq 0.05$.

P1 (*): values as compared to control group using Mann Whitney test.

P2 (♦): values as compared to breast cancer patients group before surgery using Wilcoxon signed ranks test.

P3 (¤): values as compared to breast cancer patients group without metastatic after surgery and treatment using Mann Whitney test.

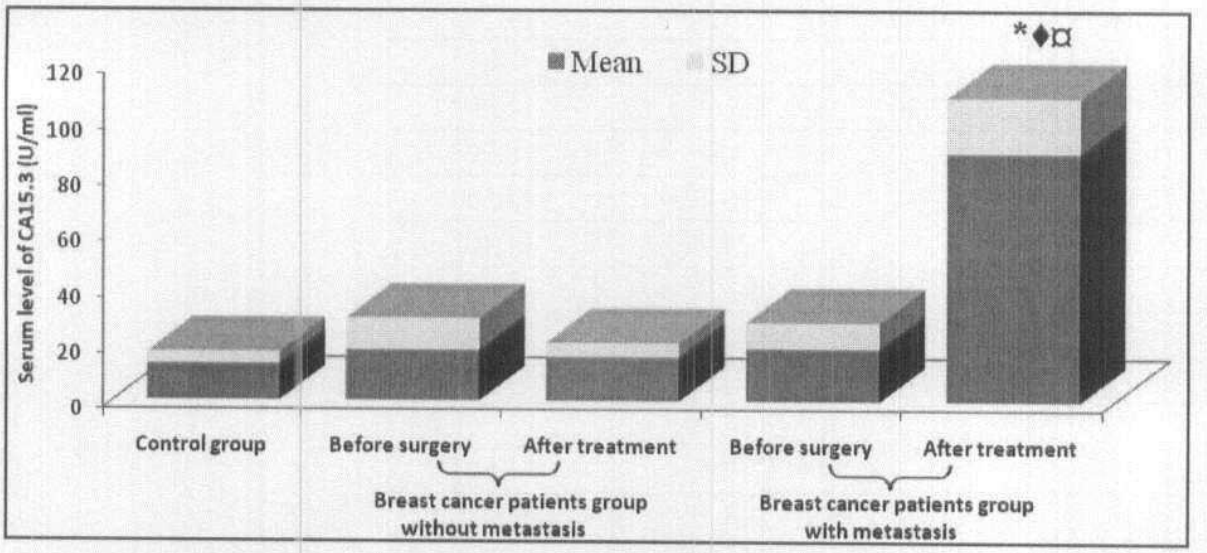


Figure (14): Serum CA15.3 (U/ml) in control group and breast cancer patients groups before surgery and after surgery and treatment

* : significantly different from control group.

♦ : significantly different from breast cancer patients groups before surgery.

¤ : significantly different from breast cancer patients group without metastatic after surgery and treatment.

Correlations between the studied biochemical parameters and clinicopathological parameters and characteristics of breast cancer patients groups:

Results of Table (11) show no correlation between big ET-1 and CA15.3 with any clinicopathological parameters and patient characteristics.

Table (11): Correlation between the studied biochemical parameters and clinicopathological parameters and characteristics of breast cancer patients groups before surgery:

Clinicopathological parameters and patient characteristics		Biochemical parameters	
		Big ET-1 (fmol/ml)	CA15.3 (U/ml)
Age	r	0.113	-0.068
	p	0.487	0.675
Tumor size	r	-0.231	0.06
	p	0.151	0.713
Lymph node	r	-0.035	0.005
	p	0.830	0.994
Pathological stage	r	0.065	0.157
	p	0.691	0.335
Histological grade	r	-0.202	-0.161
	p	0.211	0.322
Pathology type	r	0.062	0.078
	p	0.705	0.632
ER	r	-0.088	-0.107
	p	0.609	0.534
PR	r	-0.014	-0.125
	p	0.935	0.466
HER-2	r	0.109	-0.218
	p	0.656	0.369
Vascular invasion	r	-0.076	-0.178
	p	0.642	0.272
Menopausal status	r	-0.142	-0.041
	p	0.348	0.800

r: Spearman coefficient
Differences were considered statistically significant at $p \leq 0.05$.

Comparison of the diagnostic values of serum big ET-1 and CA15.3 in breast cancer patients groups before surgery using the receiver operating characteristic (ROC) curve analysis:

The ROC curve analysis was used to compare the diagnostic values of big ET-1 and CA15.3 depending on the area under the ROC curve (AUC). The higher AUC corresponds to a better diagnostic test. Serum big ET-1 showed significant AUC (100%, P<0.001) with sensitivity (80%) and specificity (100%) at a cut-off (2.0 fmol/ml). Serum CA15.3 showed insignificant AUC (66.3%, P=0.065) with sensitivity (12.5%) and specificity (100%) at a cut-off (30 U/ml).Figure (15) and Table (12).

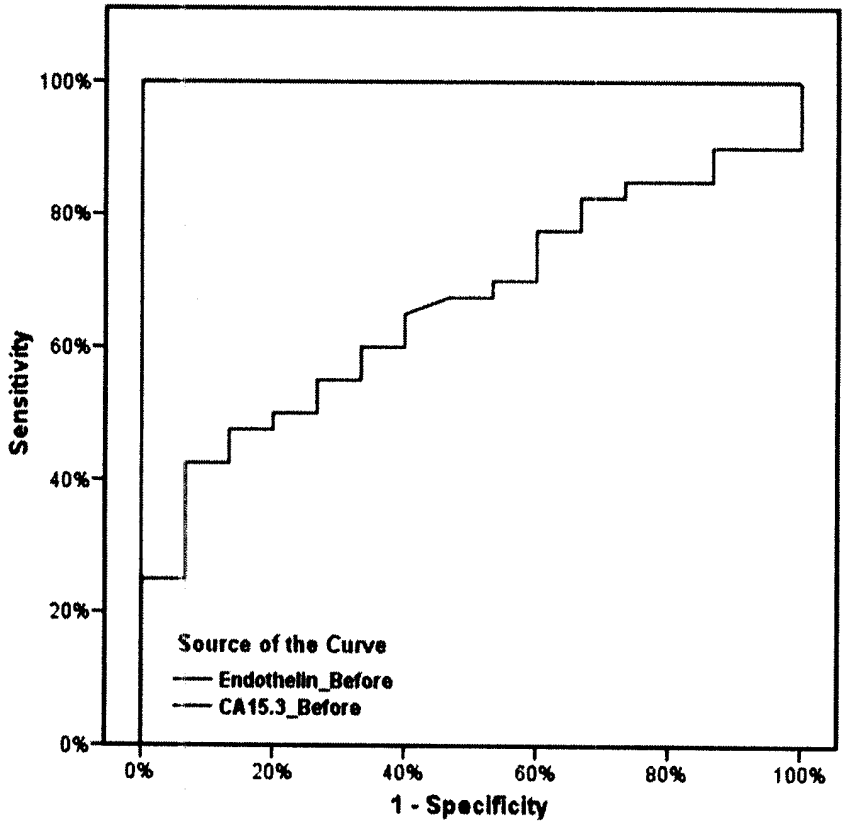
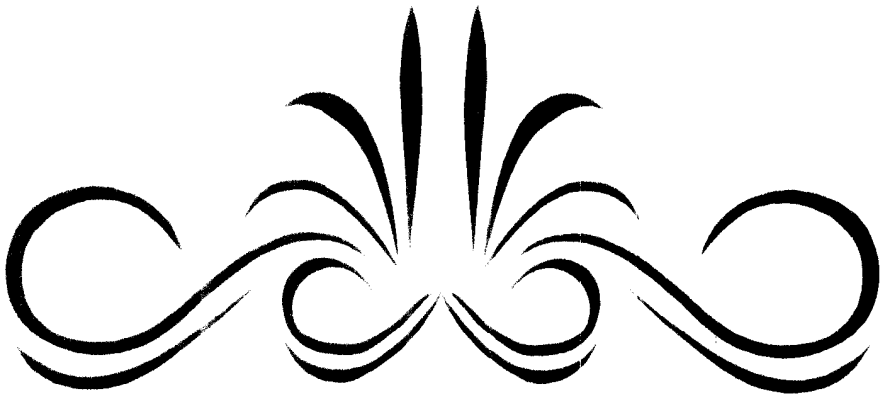


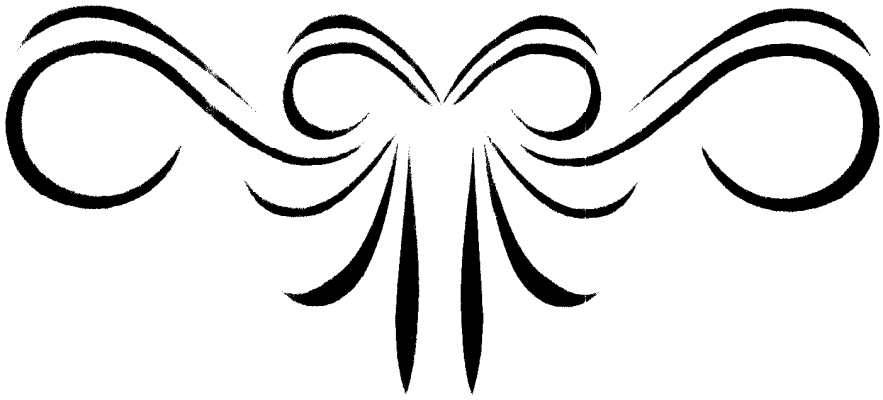
Figure (15): ROC curves for serum big ET-1 and CA15.3 in breast cancer patients groups before surgery

Table (12): **The area under the ROC curves, cut-off value, sensitivity and specificity for serum big ET-1 and CA15.3 in breast cancer patients groups before surgery**

variables	Area under the curve (%)	Asymptomatic significance (p-value)	Cut-off value	Sensitivity (%)	Specificity (%)
Big ET-1 (fmol/ml)	100	<0.001*	2.0	80	100
CA15.3 (U/ml)	66.3	0.065	30.0	12.5	100



Discussion



DISCUSSION

Breast cancer is the most common cancer among women worldwide [149,150].In Egypt, breast cancer is the most common female malignancy. In Alexandria, it accounts for about 48% of all malignancies in female [151].

The progression of breast cancer depends on the development of vascularization. In addition to the vascular endothelial growth factor pathway, the ET pathway plays a critical role in that process [152,153].

Numerous tumors produce one or more of the endothelins and their receptors, and there are many potential roles in cancer including modulating angiogenesis, inducing mitogenesis and invasive of tumor cells, and protecting cells from apoptosis [154].

Owing to the short half-life of ET-1 (3.5 minutes), however, it is thought to be a significantly less accurate marker of endothelin system activation than big ET-1[76]. Big ET-1 is a stable peptide with a half-life of (23 minutes), making the measurement of big ET-1 concentrations a sensitive indicator of endothelin system activation [75, 155].

Elevated circulating ET-1has been demonstrated in patients with breast cancer [87]. So, the aim of this study was to examine the diagnostic value of serum big ET-1, the stable precursor of ET-1, in patients having breast cancer.

The present study revealed that, serum level of big ET-1 was significantly higher in metastatic and non metastatic breast cancer patients groups before surgery than that in normal healthy control group. The biological precursor big ET-1 has a much longer half-life than ET-1 and is mainly cleared through kidney and liver [75, 76]. It has been demonstrated that human breast cancer cells express the ECE, which converts big ET-1 to ET-1 [156]. Thus, big ET-1 is also considered a candidate marker for the investigation of the ET-1 secretory activity [75]. Our observation of elevated expression of big ET-1 in breast carcinoma could potentially be attributed to the deregulation of ECE expression or activity, affecting the balance between the precursor molecule and its active form [157]. Moreover, elevated serum big ET-1 in female patients with breast cancer may be explained in view of Yildirim and his associates in (2008) who stated that the presence of the tumor causes an increase in circulating Big ET-1 levels [158].

During the follow up period (24 months), 9 patients had metastasis whereas 31 patients had no metastasis. Serum level of big ET-1 of non metastatic patients groups after surgery and treatment was significantly lower as compared with their corresponding values before surgery and become within the normal control level. Ferrari-Bravo and his associates in (2000) and Teng and his associates in (2006) reported that postoperative circulating big ET-1 level markedly decreased compared with preoperative concentrations in patients with gastric carcinoma, and concluded that ET-1 may be secreted by the cancer cell and the ET-1 concentration will fall in when the tumor is removed [159,80]. From previous observations, we can attribute the lower level of big ET-1after surgery and treatment in breast cancer patients to the removal of tumor and big ET-1 seem to change with treatment and these results are in accordance with Yildirim and his associates in (2008) who suggests that big ET-1 levels seem to change with local and systemic treatment in patients with breast cancer [158].

In our study, serum big ET-1 level of metastatic patients groups after surgery and treatment was significantly higher than control group, than their corresponding values before surgery and than breast cancer patients without metastatic after surgery and treatment. Our findings are in accordance with Yildirim and his associates in (2008) and Kalles and his associates in (2012) who reported that big ET-1 levels were significantly higher in primary or metastatic breast cancer patients compared to healthy controls [158, 160].

In keeping with the polyfunctional nature of endothelins, there are numerous potential consequences of endothelin expression in breast tumors that may lead to a more aggressive tumor cell phenotype. There is increased expression of several members of the endothelin network in invasive ductal carcinoma (IDC) of the breast when compared with the normal breast or non-invasive ductal carcinoma in situ; lymph node metastases of breast cancer have higher a higher degree of endothelin staining still [97,161]. Elevated expression of ET-1 is more common in IDCs with larger size, high histological grade and the presence of lymphovascular invasion [87], and there is increased endothelin in the serum of breast cancer patients with lymph node metastasis when compared with those with no lymph node involvement [162].

It is a known fact that tumor growth and metastasis are highly dependent upon neoangiogenesis, the formation of capillary sprouts. These may arise either from preexisting blood vessels, circulating endothelial cells, or bone marrow-derived endothelial precursor cells [163]. In addition, there is further evidence that ETs are implicated in the invasiveness of breast cancer, as the expression of ET and ETR was highest at the invasive edges of breast carcinomas, [107] and a relation between ETR expression and tumor recurrence and metastasis has been shown[87, 164]. So, we can attribute the elevation of big ET-1 in metastatic patients than non metastatic patients after surgery and treatment to increase of epithelial cancer cells.

In our study, serum levels of big ET-1 in breast cancer patients are not correlated with age, tumor size, lymph node status, pathological stage, histological grade, pathology type, hormone receptor status, vascular invasion and menopausal status. These results are in agreement with Yildirim and his associates in (2008) and Kalles and his associates in (2012) who explained lack of correlation between circulating big ET-1 and tumor size due to difference in the production and secretion rate of ET in the individual tumor [158,160].

A variety of tumor markers with varying sensitivity and specificity are used for diagnosis of different malignancies [165-167]. CA15.3 is the most widely used serum tumor marker in the screening, early detection and monitoring of treatment for breast cancer [168-171]. It is a large transmembrane glycoprotein, which is frequently over expressed [169,172-175] and aberrantly glycosylated in cancer [176].

According to our results serum CA15.3 in non metastatic patients groups either before surgery or after surgery and treatment showed insignificantly difference as compared to either control group or to each other. These results agree with previous study of Quaranta and his associates in (2007) and El Agouza and his associates in (2011) who reported that no significant differences in the serum levels of CA15.3 between breast cancer patients preoperatively and healthy controls [177, 178].

Unfortunately, CA15.3 is not suitable for early detection, as serum levels are rarely increased in patients with early or localized breast cancer. The main utility for CA15.3 is for monitoring therapy in patients with metastatic breast cancer [179].

In metastatic patients groups, CA15.3 values before surgery showed insignificantly difference as compared with control group. While after surgery and treatment, it was significantly higher than control group, than their corresponding values before surgery and than breast cancer patients without metastatic after surgery and treatment. Quaranta and his associates in (2007) found that circulating levels of CA15.3 were higher in breast cancer patients with progression or metastatic disease and these findings are in line with our results [177]. In fact, CA15.3 is elevated in only 3% of patients with localized cancer while it is elevated in up to 70% of patients with metastatic disease [180]. These elevated levels may be due to the over expression of the MUC1 gene which encodes CA15.3 [171].

Our study show no correlation between serum CA15.3 and age, tumor size, lymph node status, pathological stage, histological grade, pathology type, hormone receptor status, vascular invasion and menopausal status. These results are in accordance with Quaranta and his associates in (2007) who reported the lack of a correlation between CA15.3 and clinicopathological characteristics suggests that this cytokines are dependent on neoplasm activity and not on the stage of disease or histological type of tumor [177]. Our observation is consistent with the study done by Agyei Frempong and his associates in (2008) who reported that there was no correlation between the serum CA15.3 concentrations and age or menopausal status of breast cancer patients [169]. Also, no significant relationship between CA15.3 levels and age, menopausal status, histological type, histological grade, ER and PR was observed in study of Martín and his associates in (2006) [125]. Zheng and his associates in (2012) show no correlation between CA15.3 and menopausal status, tumor size, lymph node status, histological grade, ER, PR and HER-2 but association occur with age [181].

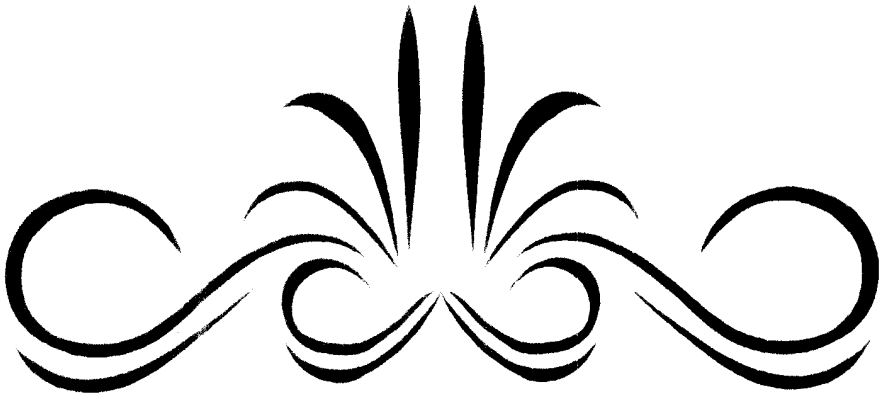
The Receiver Operating Characteristic (ROC) approaches have been extensively used in evaluating the diagnosis and prognosis power of markers [182].

The values of serum big ET-1 and CA15.3 in the early diagnosis of breast cancer in patient groups before surgery are evaluated by ROC curve analysis. The use of the area under ROC curve was useful in the elucidation of the validity of a specific marker in the early detection of breast cancer. For serum big ET-1, the area under the curve (AUC) = 100% indicating the validity of using big ET-1 as a marker for early diagnosis of breast cancer patients. While for CA15.3, AUC = 66.3 % revealed the rejection of using CA15.3 in diagnosis of breast cancer patients.

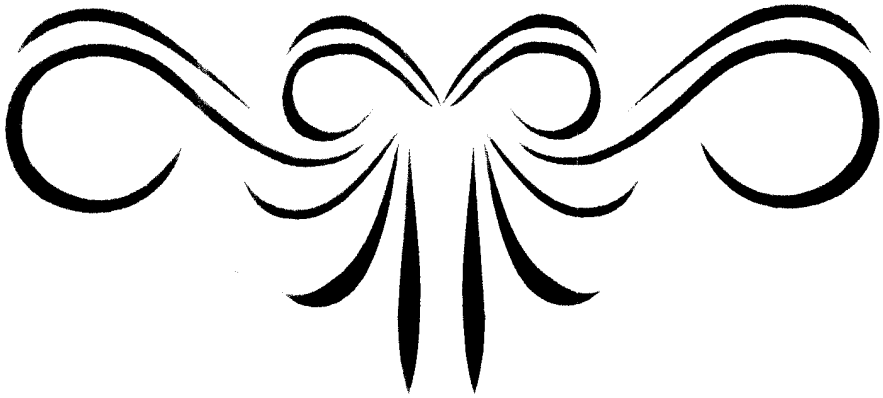
The impressive finding is the sensitivity and specificity of big ET-1 at the estimated cut-off value of 2.0 fmol/ml was 80 and 100% respectively. On the other hand the estimated cut-off value of CA15.3 was 30 U/ml at this level the sensitivity and specificity was 12.5 and 100% respectively, and this finding agree with previous studies emphasized that CA15.3 lacking sensitivity for early disease of breast cancer [181,183]. From the research of Keyhani and his associates in (2005), sensitivity and specificity of serum CA15.3 were 14 and 92.3% using cut-off value of 30 U/ml [183]. However, Agyei Frempong and his associates in (2008) reported the sensitivity and specificity of serum CA15.3 in detecting breast cancer using cut-off value of 35 U/ml was 76.1 and 100% respectively. This disparity may have occurred due to the different assay procedure and a cut-off value [169].

Based on these finding, we can suggest that big ET-1 is more efficient in preliminary screening of breast cancer. While CA15.3 has no place in screening for early diagnosis of breast cancer [112].

To the best of our knowledge, this is the first study that compares the diagnostic value of serum big ET-1 with that of serum CA15.3 by determination of cut-off value, sensitivity and specificity of each marker in breast cancer patients.



Summary



SUMMARY

Breast cancer is the most prevalent cancer among women. Like all malignancies, breast cancer arises as a result of the accumulation of genetic alterations, especially deregulation of the expression of oncogenes and tumor suppressor genes.

Tumor markers are widely used in the management of patients with breast cancer, during therapy for metastatic disease and in conjunction with diagnostic imaging, history and physical examination.

Big endothelin-1 (BigET-1), the biological precursor of endothelin-1 (ET-1) is a more accurate indicator of the degree of activation of the endothelial system compared to ET-1due to it has a longer half-life and slower clearance than ET-1. In breast cancer, expression of components of the endothelin system has been associated with the transition from normal tissue to progressively invasive lesions.

CA15.3 is the most widely used serum marker for breast cancer, it is recommended in the evaluation of response to therapy and for monitoring the course of breast cancer.

The aim of the present study was to evaluate the diagnostic value of serum big ET-1 in comparison with serum CA15.3 in breast cancer patients. The present study was carried out on 55 females which are divided into Control group: included 15 normal healthy females and malignant group: included 40 female patients with newly diagnosed breast cancer of different clinical stages. They were included non metastatic patients (n=31) and metastatic patients (n=9) which detected during follow up. Blood samples were with drawal from all patients before surgery, after both surgery and adjuvant treatment protocols and from control subjects. Serum big ET-1 levels were measured in all studied group by ready-to-use Enzyme Immunoassay (EIA) kit. Serum CA15.3 was measured using ready-to-use immunoradiometric assay (IRMA) kit.

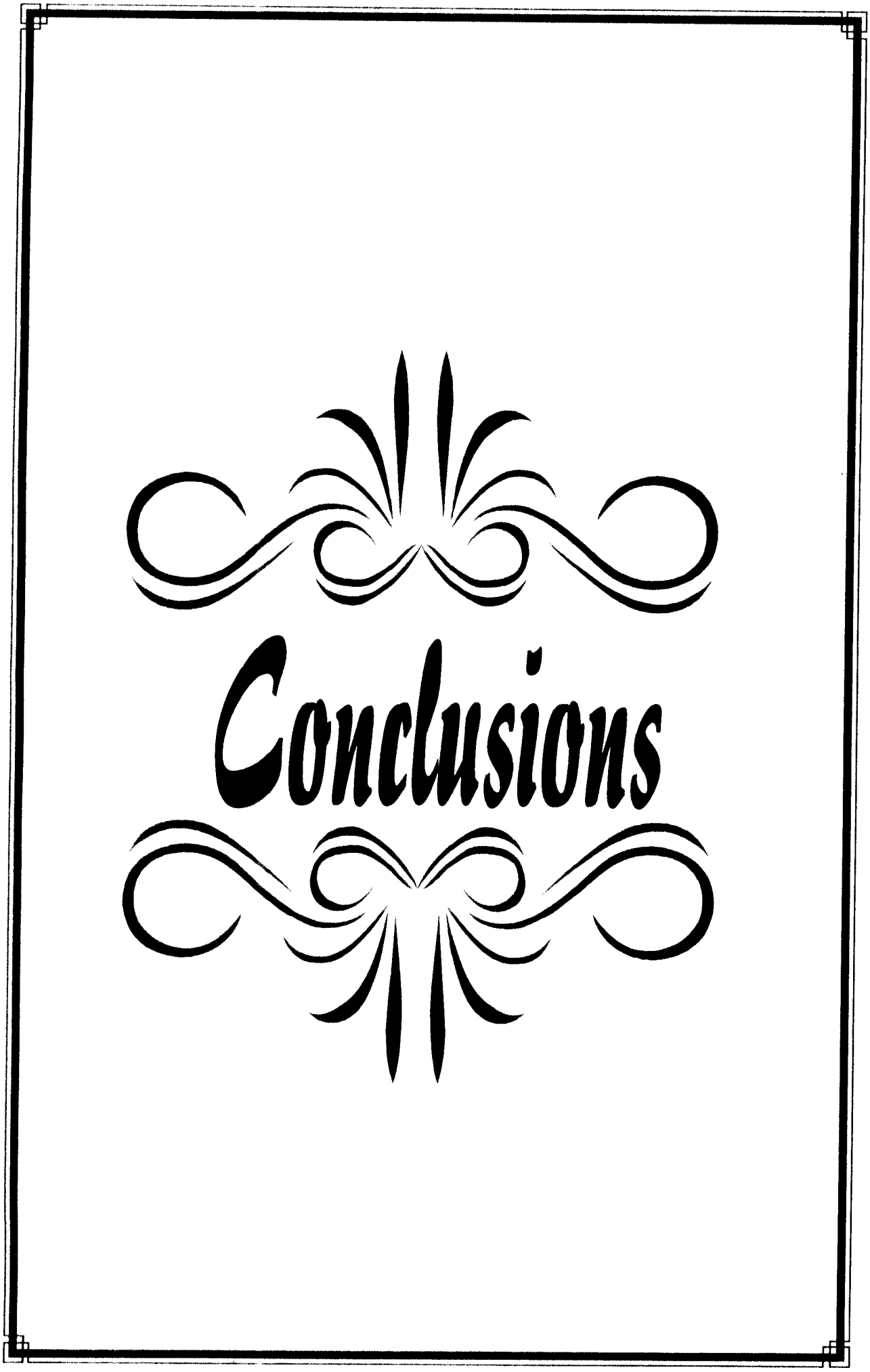
Serum level of big ET-1 was significantly higher in metastatic and non metastatic breast cancer patients before surgery than in normal healthy control. Elevated level of big ET-1 in breast carcinoma could potentially be attributed to the deregulation of ECE expression or activity, affecting the balance between the precursor molecule and its active form. Serum level of big ET-1 of non metastatic patients after surgery and treatment was significantly lower as compared with their corresponding values before surgery and become within the normal control level. Lower level of big ET-1 in non metastatic breast cancer patients after surgery and treatment may be due to the removal of tumor and big ET-1 level seems to change with treatment. In addition, serum big ET-1 level of metastatic breast cancer patients after surgery and treatment were significantly higher than control group, than their corresponding values before surgery and than breast cancer patients without metastatic after surgery and treatment. Higher level of big ET-1 in metastatic breast cancer patients after surgery and treatment may be due to increase in epithelial cancer cells.

According to the results of our study, serum CA15.3 in non metastatic patients either before surgery or after surgery and treatment showed insignificantly difference as compared to either control group or to each other. CA15.3 levels are rarely increased in patients with early or localized breast cancer. The main utility for CA15.3 is for monitoring

therapy in patients with metastatic breast cancer. In metastatic patients, CA15.3 value before surgery showed insignificantly difference as compared with control group. While after surgery and treatment, it was significantly higher than control group, than their corresponding values before surgery and than breast cancer patients without metastatic after surgery and treatment. These elevated levels may be due to the over expression of the MUC1 gene which encodes CA15.3. Our study show no correlation between serum big ET-1 or CA15.3 and age, tumor size, lymph node status, pathological stage, histological grade, pathology type, hormone receptor status, vascular invasion and menopausal status.

The ROC curve analysis was applied to comparing the diagnostic values of these markers and to determine which one is more reliable for the early diagnosis of breast cancer. Area under the curve was 100% for big ET-1 and 66.3% for CA15.3. The optimum cut-off value selected for big ET-1 was 2.0 fmol/ml, at which the sensitivity was 80% and specificity was 100%. While for CA15.3 the optimum cut-off value was 30 U/ml, at which the sensitivity was 12.5% and specificity was 100%. So, it was found that serum big ET-1 is diagnostic marker for breast cancer. However, rejection the using of CA15.3 in diagnosis of breast cancer patients.

To the best of our knowledge, this is the first study that compares the diagnostic value of serum big ET-1 with that of serum CA15.3 by determination of cut-off value, sensitivity and specificity of each marker in breast cancer patients.


Conclusions

CONCLUSIONS

From our results, we can conclude that:

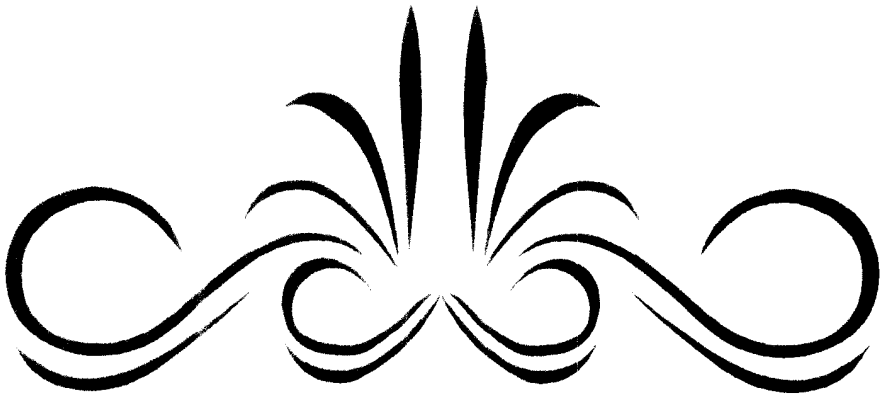
1- Serum big ET-1 may provide a useful tool for the early diagnosis of breast cancer patients.

2- Serum CA15.3 was found to have no value in the screening for early diagnosis of breast cancer patients.

3- Both serum big ET-1 and CA15.3 can be used as potential factors to predict of metastatic breast cancer patients during active therapy.

So, we can recommend that:

Further studies with larger number of patients are recommended to demonstrate the prognostic importance of serum big ET-1 in patients with breast cancer.



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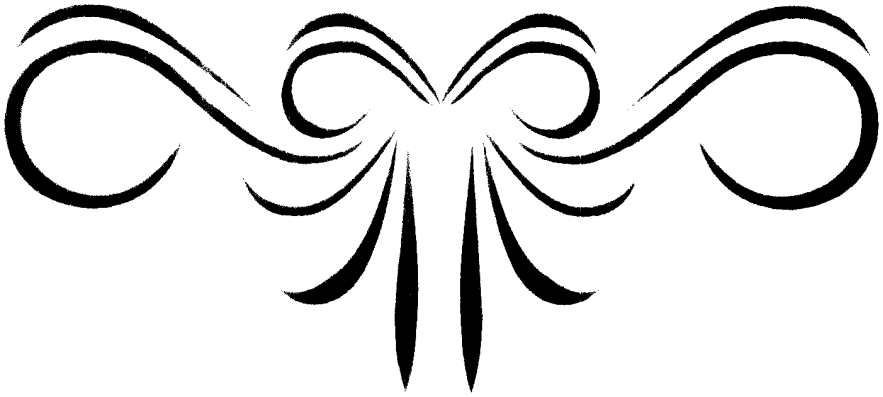

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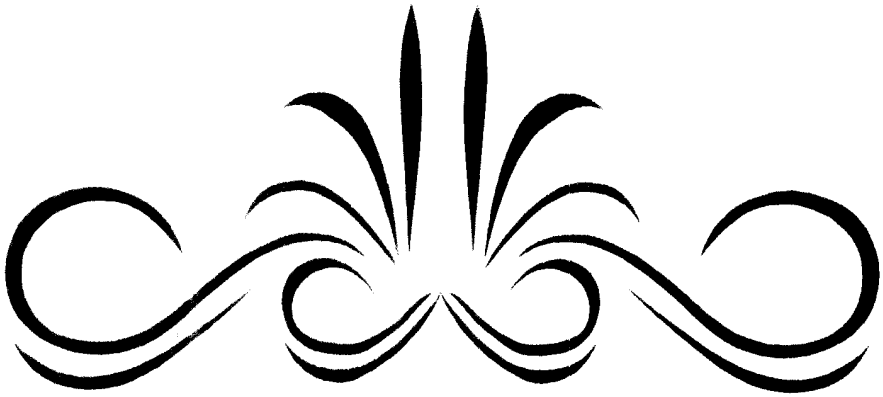
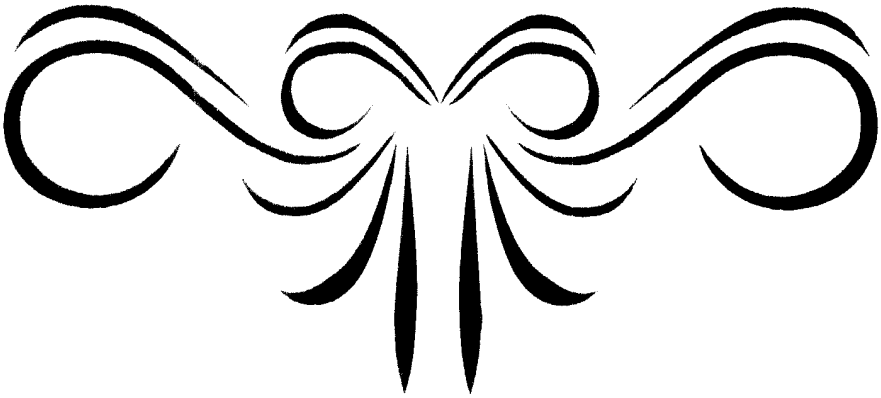


Protocol



دراسة اندوثلين-١ الكبير كاحد دلالات الاورام فى مريضات سرطان الثدى المصريات

Study of big endothelin-1 as a tumor marker in Egyptian female patients with breast cancer

خطة بحث مقدمة الى
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الحصول على درجة

Protocol of a thesis submitted to the
Medical Research Institute
University of Alexandria
in partial fulfillment of the
requirements of the degree of

الماجستير فى الكيمياء الطبية التطبيقية

Master of Applied Medical Chemistry

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Background

Breast cancer is the most common cancer among women and is the second leading cause of cancer –related death throughout the world. [1]

Nowadays, early prevention and detection of this malignancy is urgently needed since a lot of patients succumb to advanced disease. Current methods used to detect breast cancer tumors are based on mammography which is a clinically screening method effective in detecting breast cancer before clinical symptoms appear. However, since a tumor should be at least a few millimeters in size, it is already late when breast cancer is detected. Hence, there is a considerable need for the identification of useful pathological markers of this disease that can help not only in detection but also for typing and treatment. [2]

Tumor markers could identify a disease process and specific tissue or patient's characteristics, and help establishing the severity and extend of the disease. They are usually not used alone for the diagnosis because most markers can be found in elevated levels in people who have benign conditions, and because no tumor marker is yet specific to a particular cancer. [3]

CA15-3 (cancer antigen 15-3) is one of the most reliable tumor markers used in monitoring breast cancer patients. [4] It has been reported that the sensitivity and specificity of serum CA15-3 for detecting metastatic diseases are higher than those of CEA (carcinoembryonic antigen) however; the sensitivity of the former in detecting metastatic diseases has been reported to be limited to 63%. Several tumor markers have been reported to be complementary to serum CA15-3 in detecting metastatic diseases. [5, 6]

Endothelins (ETs), including ET-1, ET-2, ET-3, are small 21-residue peptides.[7] There are at least two receptor subtypes, ETAR (endothelin A receptors) and ETBR (endothelin B receptors), belonging to the family of G-protein-linked receptors with seven transmembrane spanning domains.[8] The ET-1 gene encodes a precursor peptide, preproendothelin-1, which is cleaved by a neutral endopeptidase to form proendothelin-1 or big endothelin-1.Due to a low circulating concentration and short serum half life (about1.5 min), measurement of serum ET-1 concentrations has proven to be difficult. Big ET-1 is a stable peptide with a serum half life of 30 minutes, making the measurement of plasma big ET-1 concentrations a sensitive indicator of endothelin system activation.[9, 10]

ET-1 is a relevant growth factor in several tumor types including carcinoma of the prostate, ovary, colon, cervix, breast, kidney, lung, colon, central nervous system tumors as well as melanoma, Kaposi's sarcoma and bone metastasis.[11] Recent studies have suggested that ET-1 play an important role in tumorigenesis, tumor progression and metastasis presumably by various mechanisms, including mitogenesis, inhibition of apoptosis, angiogenesis and mediating extracellular matrix degradation.[12-16]

ET-1 and its receptors, ETAR and ETBR are over expressed in breast carcinomas. ET-1 released from breast cancer cell binds ETAR on breast fibroblasts. It was suggested that breast fibroblasts stimulate breast cancer cells by paracrine manner.[15] ET-1, ETAR and ETBR expression was also associated with increased VEGF (vascular endothelial growth factor) expression and higher vascularity.[14] Therefore analysis of the ET-axis and in particular of ETAR may improve the prediction of relapse and death and may identify patients who may profit from ETAR targeted adjuvant therapy.

Aim of the study

In this study, we will investigate serum big ET-1 levels in patients with breast cancer and its correlation with CA15.3, clinical and pathological criteria as well as outcome of the disease.

Subjects and methods

The study will include 55 females divided into 2 groups:

- Malignant group: will include 40 female patients with newly diagnosed breast cancer of different clinical stages.
- Control group: will include 15 normal healthy age-matched females.

All patients will be selected from those admitted to the Surgery and Oncology Departments at Medical Research Institute, Alexandria University.

Patients of malignant group will be treated by surgical technique, followed by an adjuvant treatment protocol according to the stage of cancer.

Two blood samples will be taken from each patient of malignant group: Before surgery and after both surgery and adjuvant treatment protocol. Only one blood sample will be taken from each subject of the control group.

All blood samples will be collected in specimen tubes and will be centrifuged; serum supernatant will be collected and stored at -70°c until use for determination of:

1- Big endothelin-1 level with an enzyme immunoassay kit without extraction. [17]

2- CA 15.3 level (a standard prognostic marker) with radio-immunoassay. [18]

Analysis of Results

SPSS, software will be used for statistical analysis. The Chi – square and Mann-Whitney U tests will also be used.

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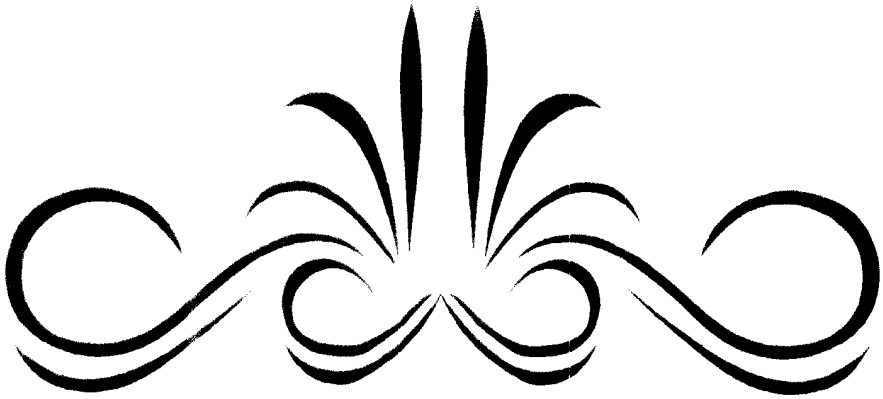
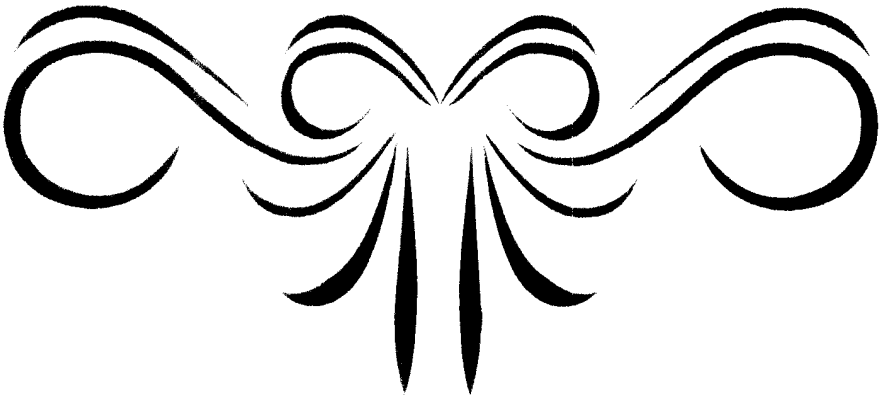


Arabic Summary



الملخص العربي

الملخص العربى

دراسة اندوثلين -١ الكبير كأحد دلالات الأورام فى مريضات سرطان الثدى المصريات

سرطان الثدى هو أكثر أنواع السرطان انتشارا" بين النساء. سرطان الثدى، مثل كل الأورام الخبيثة ينشأ نتيجة لتراكم التغيرات الجينية، ولا سيما التغير فى التعبير عن الجينات المسرطنة والجينات المثبطة للورم.

دلالات الأورام تستخدم على نطاق واسع فى تحديد المرضى الذين يعانون من سرطان الثدى، خلال علاج المرض المنتشر وبالتزامن مع التصوير التشخيصى والتاريخ المرضى والفحص البدنى.

الأندوثلين-١الكبير هو السالف البيولوجى للأندوثلين-١ يعتبر مؤشر أكثردقة لمعرفة درجة نشاط نظام الأندوثلين مقارنة بالأندوثلين-١، نتيجة الى انه يملك فترة نصف عمر أطول والتخلص منه ابطأ من الأندوثلين-١. فى سرطان الثدى التعبير عن مكونات نظام الأندوثلين ارتبطت بالتحول من الأنسجة الطبيعية إلى أورام أكثر هجوما" تدريجيا".

مصل الأنتجين الكربوهيدراتى١٥.٣ هو أكثر دلالات الأورام استخداما" لسرطان الثدى، وهو مستحسن فى تقيم الأستجابة للعلاج ورصد مسار سرطان الثدى.

الهدف من الدراسة:

الهدف من هذه الدراسة لتقدير القيم التشخيصية للأندوثلين-١ الكبير بالمقارنة مع الأنتيجن الكربوهيدراتى ١٥.٣ فى مصل مريضات سرطان الثدى.

المرضى والطرق المعملية المستخدمة فى البحث:

وقد أجريت هذه الدراسة على ٥٥ من الإناث التى تم تقسيمها إلى مجموعة الأصحاء والتى تشمل ١٥ من الإناث الصحية الطبيعية ومجموعة مريضات سرطان الثدى والتى شملت ٤٠ مريضة من الإناث المصابات بسرطان الثدى والتى تم تشخيصها حديثا" من مراحل سريرية مختلفة. تم ادراجهم إلى مريضات سرطان غير منتشر (عددهم=٣١) ومريضات سرطان منتشر (عددهم=٩) والتى تم الكشف عنهم خلال المتابعة. عينات الدم تم سحبها من جميع المريضات قبل الجراحة وبعد كلا" من الجراحة وبروتوكول العلاج ومن مجموعة الأصحاء. تم قياس مستوى مصل الأندوثلين-١ الكبير فى جميع مجموعات الدراسة باستخدام طريقة القياس المناعى الأنزيمى المعدة للاستخدام المباشر. وتم قياس مستوى مصل الأنتيجن الكربوهيدراتى ١٥.٣ باستخدام طريقة القياس المناعى الأشعاعى المعدة للاستخدام المباشر.

النتائج والمناقشة:

مستوى مصل الأندوثلين-١ الكبير به زيادة ذو دلالة احصائية فى كلا" من مريضات سرطان الثدى المنتشر وغير المنتشر قبل الجراحة مقارنة بمجموعة الأصحاء. المستوى المرتفع للأندوثلين-١ الكبير يمكن ان يرجع الى التغير فى التعبير عن او نشاط انزيم الأندوثلين المتحول والتى تؤثر على التوازن بين الجزىء السالف وشكله النشط.

مستوى مصل الأندوثلين-١ الكبير فى مريضات سرطان الثدى الغير منتشر بعد كلا" من الجراحة والعلاج به انخفاض ذو دلالة إحصائية بالمقارنة مع ما يقابلها من القيم قبل الجراحة وأصبحت فى المستوى الطبيعى للأصحاء. انخفاض مستوى الأندوثلين-١ الكبير بعد الجراحة والعلاج فى مريضات سرطان الثدى يرجع إلى إزالة (استئصال) الورم ويبدو أن مستوى الأندوثلين-١ الكبير يتغير مع العلاج . بالإضافة إلى ذلك، مستوى مصل الأندوثلين-١ الكبير فى مريضات سرطان الثدى المنتشر بعد الجراحة والعلاج كان به زيادة ذو دلالة احصائية بالمقارنة بكلا" من مجموعة الأصحاء، ما يقابلها من القيم قبل الجراحة و مريضات سرطان الثدى الغير منتشر بعد كلا" من الجراحة والعلاج. يمكن أن ننسب ذلك إلى زيادة الخلايا السرطانية الظهارية.

وفقا لنتائج دراستنا، أظهرت ان الأنتجين الكربوهيدراتى ١٥.٣ فى مصل مريضات سرطان الثدى غير المنتشر إما قبل الجراحة اوبعد الجراحة والعلاج به اختلاف معنوى بالمقارنة إما بمجموعة الأصحاء أو ببعضهم.

نادرا" ما تزيد مستويات الأنتجين الكربوهيدراتى ١٥.٣ فى مصل مريضات سرطان الثدى المبكر أو الموضعى. الأستخدام الأساسى للأنتجين الكربوهيدراتى ١٥.٣ هو لرصد العلاج فى مرضى سرطان الثدى المنتشر.

فى مريضات سرطان الثدى المنتشر،أظهرت قيم الأنتجين الكربوهيدراتى١٥.٣ قبل الجراحة اختلافا" معنويا" مقارنة بمجموعة الأصحاء. بينما بعد الجراحة والعلاج، كان به زيادة ذو دلالة احصائية بالمقارنة بكلا" من مجموعة الأصحاء، ما يقابلها من القيم قبل الجراحة و مريضات سرطان الثدى الغير منتشر بعد كلا" من الجراحة والعلاج. قد تكون هذه المستويات المرتفعة نتيجة التعبير الزائد لجين الميوسين١ الخاص بالأنتجين الكربوهيدراتى ١٥.٣.

أظهرت دراستنا عدم وجود علاقة بين الأندوثلين-١ الكبير أو الأنتجين الكربوهيدراتى ١٥.٣ والعمر، حجم الورم، حالة الغدد الليمفاوية، المراحل السريرية ، حالة مستقبلات الهرمون، غزو الاوعية الدموية وحالة أنقطاع الطمث فى مريضات سرطان الثدى.

منحنى ROC تم تطبيقه لمقارنة القيم التشخيصية لتلك الدلائل لتحديد إيهم يمكن أن يعتمد عليه فى التشخيص المبكر لسرطان الثدي. المساحة تحت المنحنى كانت ١٠٠% للأندوثلين-١ الكبير و ٦٦.٣% للأنتجين الكربوهيدراتى١٥.٣ . قيمة المعيار المثالية التى تم اختيارها للأندوثلين-١ الكبير كانت ٢.٠ فيمتو مول/ مل، وكانت عندها الحساسية ٨٠% والخصوصية ١٠٠%. قيمة المعيار المثالية للأنتجين الكربوهيدراتى١٥.٣كانت ٣٠ وحدة/ مل، وكانت عندها الحساسية١٢.٥% والخصوصية ١٠٠%. وبالتالى وجد ان الأندوثلين-١ الكبير دليل تشخيصى لسرطان الثدى. واستبعاد استخدام الأنتجين الكربوهيدراتى ١٥.٣ فى تشخيص مرضى سرطان الثدى.

يصل الى حد علمنا، أن هذه هى أول دراسة تقارن القيم التشخيصية للأندوثلين-١ الكبير بالأنتجين الكربوهيدراتى١٥.٣ عن طريق تحديد قيمة المعيار المثالية، الحساسية والخصوصية لكلا" منهم فى مريضات سرطان الثدى.

<u>من نتائجنا، يمكننا ان نستنتج ما يلى:</u>

١- مصل الأندوثلين-١ الكبير ممكن أن يكون أداة مفيدة للتشخيص المبكر لمرضى سرطان الثدى.

٢- مصل اللأنتجين الكربوهيدراتى١٥.٣ وجد أنه ليس له قيمة فى التشخيص المبكر لمرضى سرطان الثدى.

٣- يمكن أستخدام كلا" من مصل الأندوثلين-١ الكبير والأنتيجين الكربوهيدراتى ١٥.٣ كعوامل محتملة للتنبؤ بمرضى سرطان الثدى المنتشر خلال العلاج .

<u>لذلك، يمكن أن نوصى بالآتى:</u>

المزيد من الدراسات مع عدد أكبر من المرضى يوصى به لتحديد الأهمية التنبؤية للأندوثلين-١ الكبير فى مرضى سرطان الثدى.

لجنة الإشراف

موافقون

الأستاذة الدكتورة/ منى محمد رشاد
أستاذ متفرغ بقسم الكيمياء الطبية التطبيقية
معهد البحوث الطبية
جامعة الإسكندرية

.....

الأستاذ الدكتور/ ياسر مصطفى القرم
أستاذ مساعد بقسم علاج وأبحاث الأورام
معهد البحوث الطبية
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الأستاذ الدكتور/ مدحت محمد أنور
أستاذ مساعد بقسم الجراحة الإكلينيكية والتجريبية
معهد البحوث الطبية
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الدكتورة/ نيفين عبد المنعم حسين
مدرس بقسم الكيمياء الطبية التطبيقية
معهد البحوث الطبية
جامعة الإسكندرية

.....

دراسة اندوثلين -١ الكبير كأحد دلالات الأورام فى مريضات سرطان الثدى المصريات

مقدمة من

نورهان أحمد أبوزيد على

بكالوريوس علوم (كيمياء – كيمياء حيوية) – جامعة الإسكندرية ٢٠٠٤

دبلوم كيمياء حيوية – جامعة الإسكندرية ٢٠٠٥

للحصول على درجة

الماچستيير

فى

الكيمياء الطبية التطبيقية

لجنة المناقشة والحكم على الرسالة **موافقون**

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الأستاذ الدكتور/ محمد نجيب دسوقى
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كلية الطب
جامعة الإسكندرية

الأستاذ الدكتور/ ياسر مصطفى القرم
أستاذ مساعد بقسم علاج وأبحاث الأورام
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دراسة اندوثلين ـ١ الكبير كأحد دلالات الأورام فى مريضات سرطان الثدى المصريات

رسالة علمية
مقدمة إلى معهد البحوث الطبية- جامعة الإسكندرية
إيفاءاً جزئياً لشروط الحصول على درجة

الماچستيير

فى

الكيمياء الطبية التطبيقية

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٢٠١٢