Osteopontin Expression in Breast Cancer Patients and Healthy Individuals

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(Molecular Biology)

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Dedication

This thesis is dedicated to:

- The soul of my father
- My merciful mother
- My supportive and beloved husband
- My beautiful angels Maryam and Mina
- My dear brother and sister
- All who are suffering from cancer

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Lina

Abstract

Breast cancer is one of the main causes of morbidity and mortality in women around the world. The primary factor that contributes to breast cancer mortality is the existence of metastasis, which is the leading cause of death that accounts for more than 400,000 deaths annually all over the world. Therefore, it is important to identify new biomarkers with the potential to develop early diagnosis and to predict patient prognosis, drug resistance improvement and treatment choice. While biomarkers for particular types of cancer have importance, markers that detect multiple cancers may be more valuable in diagnostic screening, where it is impractical to have one marker for every possible cancer.

Osteopontin is a phosphorylated glycoprotein that is capable of binding to cell surface integrin, and due to its adhesive properties, it has been associated with the progression of more than 30 types of cancer. The marker has been determined in 60 samples from Kurdistan region/ Sulaimani/ Iraq, including 35 blood samples from females with breast cancer, blood samples from 10 healthy women and 15 tissue samples from women referred for core biopsy diagnosis following an initial suspicious screening mammogram. Osteopontin mRNA expression levels were measured using semi-quantitative reverse transcriptase-polymerase chain reaction sq-RT-PCR. The results of data analysis showed that osteopontin was expressed in all the samples, with different expression levels. It was noticed that there was a significant difference between the levels of osteopontin mRNA expression in the blood of breast cancer patients compared to healthy samples ($P \leq 0.05$); this correlated with a significant decrease in osteopontin expression levels after the first cycle of chemotherapy compared with same samples before chemotherapy ($P \leq 0.05$).

The levels of the osteopontin-c mRNA from tissue samples were highly significant between Grade I, and Grade III in breast cancer patients ($p = 0.021$),

while there was no significant difference between Grade I and Grade II ($p > 0.05$). It was also noticed that the expression levels of osteopontin-c mRNA elevated in the blood of breast cancer patients compared to healthy control women ($p = 0.011$).

The main important part of this study was the comparison between Osteopontin-c mRNA expression levels in both tissue and blood from the same breast cancer patients that showed no significant correlation between the two groups ($p = 0.641$). The results of the present study suggest that gene expression of osteopontin may help as a possible diagnostic and prognostic tool for breast cancer monitoring and osteopontin-c can be regarded as a selective marker for breast cancer as it is more reliable than osteopontin because osteopontin –c is absent in normal breast tissues.

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List of Abbreviations

Abbreviations	Meaning
µl	Micro liter
18S rRNA	18S ribosomal RNA
BME	β-mercaptoethanol
bp.	Base pair
BRCA1	breast cancer 1
BRCA2	breast cancer 2
BRIP1	BRCA1 interacting protein C-terminal helicase 1
BSP	bone sialoprotein
CBE	Clinical breast examination
CDH1	Cadherin-1
CDKN2A	cyclin-dependent Kinase Inhibitor 2A
cDNA	complementary DNA
CHECK2	checkpoint Kinase 2
CT	Ultrasound tomography scan
DCIS	Ductal carcinoma in situ
DMP	Dentin matrix protein
DNA	deoxyribonucleic acid
dNTP	deoxy nucleoside triphosphate
DSA	DNase Stop Solution
DSPP	Dentin sialophospho protein
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid

EGFR	Epidermal growth factor receptor
ELISA	enzyme-linked immune sorbent assay
ER	Estrogen receptors
erbB2+	Receptor tyrosine Kinase 2.
ERK1/2	Extracellular signal-regulated kinases
Eta-1	early T-lymphocyte activation -1-
FFDM	full-field digital mammography
FNA	Fine needle aspiration
GAPDH	Glyceraldehyde-3-phosphatedehydrogenase
HER2	human epidermal growth factor receptor 2
KDa.	Kilo Dalton
Kb	Kilobase
LR	local recurrence
mRNA	Messenger ribonucleic acid
MEPE	Matrix extracellular phosphor glycoprotein
mg	Milligrams
ml	Milliliter
MMP-9	matrix metalloproteinase 9
MRI	Magnetic resonance imaging
NC	Negative control
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSCLC	Non-Small Cell Lung Cancer
OPN	Osteopontin
PALB2	Partner and localizer of BRCA2
PCR	Polymerase chain reaction

PGK	Phosphoglycerate Kinase
PR	Progesterone receptor
PTMs	Post translation modifications
Rb1	Retinoblastoma 1
RBC	Red blood cells
RNA	Ribonucleic acid
RPL-19	Ribosomal protein 19
RT-PCR	Reverse transcriptase polymerase chain reaction
Runx2	Runt-related transcription factor 2
RWA	RNA Wash Solution
SFM	Screen-film mammography
SIBLING	Small integrin –binding ligand N-linked glycoprotein
SPP-1	Secreted phosphoprotein 1
STAT-3	Signal transducer and activator of transcription 3
TAE	Tris-acetate-EDTA
TBE	Tris/Borate/EDTA
TP53	Tumor protein p53
UV	Ultraviolet
V	Volt
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
Vol	Volume
WHO	World Health Organization
ACTB	B-actin
CETN2	Centrin-2

HKGs	Housekeeping genes
ESCC	Esophageal squamous cell carcinoma

Chapter One

Introduction

Chapter one

Introduction

1.1 Introduction

Breast cancer is considered as the most common cancer type in women with over 1.6 million cases diagnosed annually [187]. The survival rate of breast cancer patients reported to be 100% for early stages (zero and one), and for stage four is just about 20% for five years survival [200], this confirms the importance of early detection, so treatment initiation can be as soon as possible during tumor development [101]. Biomarkers have enhanced the ability for diagnosis, prognosis as well as therapy prediction. In general, a suitable biomarker should be useful in defining risks and identifying the early stages of cancer development [107].

OPN was initially described by Senger *et al*. (1979) as a transformation associated phosphoprotein; it was later named secreted phosphoprotein 1 (SPP-1) [47]. It is a secreted phosphoprotein characterized as a biomarker of tumor metastasis because increased OPN expression is found within tumor cells. Nowadays, OPN is considered as a serum biomarker for predicting tumor metastasis [206]. OPN level can be a useful biochemical marker easily calculated in plasma of breast cancer patients to provide early signals for metastases [143]. It is involved in most aspects of tumor biology. Through its diverse functions related to proliferation, survival, angiogenesis, tumor development, invasion, and metastasis, OPN covers multiple hallmarks of cancer. Indeed, OPN expression significantly correlates with tumor stage in various cancers types [145].

Recent studies report that OPN overexpression has been detected in many human carcinomas, for example, lung, breast, gastric, colorectal cancer, and hepatocellular carcinoma. It was suggested that OPN levels in blood or tumor samples may be useful for predicting the prognosis of carcinomas, and the inhibition of OPN might

be helpful for the treatment of patients with carcinoma [255]. Alternative splicing of osteopontin mRNA leads to 3 isoforms, OPN-a, OPN-b, and OPN-c [212]. Osteopontin-c is a diagnostic and also a prognostic marker that has importance in a diagnostic panel together with conventional breast cancer markers [135].

1.2 The aim of the study

To investigate the role of *osteopontin* gene expression as a possible diagnostic and prognostic tool for breast cancer monitoring

1.3 Thesis objectives

- To evaluate the *Osteopontin* mRNA expression levels by Sq-RT-PCR in breast cancer patient's blood and to compare the expression levels of *OPN* before and after one cycle of chemotherapy.
- To compare the levels of *OPN* among patients with breast cancer and healthy women
- To study the mRNA expression levels of *OPN –c* from both blood and tissue samples to determine the diagnostic and prognostic role of *OPN-c* isoforms.
- To study the difference in *osteopontin* gene expression between the human breast tissue and the blood from the same samples.

Chapter Two

Literature Review

Chapter two
Literature Review

2.1 History of cancer

Cancer is a genetic disease that arises from an accumulation of mutations in various genes. It is defined as a malignant (tumor), characterized by an abnormal growth and unregulated proliferation of cells that have the ability to invade surrounding tissues and metastasize to distant sites [78]. Healthy cells are always subject to signals that order whether the cell should divide, differentiate into another cell or die. Cancer cells develop a degree of self-sufficiency from these signals, resulting in uncontrolled growth and proliferation [83]. Carcinogenesis refers to the process by which genetic mutations accumulate in healthy cells. The process by which a typical cell becomes a tumor is called carcinogenesis or tumorigenesis [150]. The recent theory is that cancer is the result of both genetic and environmental influences that changes gene expression leading to uncontrolled cell growth [96]. Although cancer encompasses many different tumor types, classified by their cellular origin, they all share common hallmarks that involve some biological capabilities acquired throughout the multistep progress of human tumors [79].

Hanahan and Weinberg in 2011 described the six hallmarks to become a cancer cell: (1) sustaining proliferative signaling. (2) Escaping growth suppressors. (3) Resisting cell death (apoptosis), (4) Supporting the replicative immortality, (5) Inducing Angiogenesis, and (6) Activation of invasion and metastasis. A typical cell needs two essential characteristics to develop into a cancer cell and to promote tumor progression which is genomic instability and the inflammatory state of premalignant and malignant lesions [79].

Figure 2-1: The six hallmarks of cancer [79].

2.1.1 Cellular basis of cancer

The majority of tumors can be classified based on their tissue site of origin. The most common form of cancer which accounting for over 80% of all cancers arises in epithelial cells, these tumors is termed carcinomas. The majority of carcinomas may split into squamous cell carcinomas or adenocarcinomas; it depends on whether the epithelial cells of origin are part of the protective epithelial layer (squamous cell carcinomas), or have secretory properties named adenocarcinomas [236]. After carcinomas, sarcomas arise from mesenchymal cells such as connective tissue, adipocytes or osteoblasts. Hematological malignancies can split

into leukemia and lymphoma; leukemia results from various lineages of white blood cells and lymphomas derived from lymphoid lineages that go on to form hard tumor masses. Among others, there are a small number of other cancers, such as melanomas, which do not fit into any of the above category [236].

2.1.2 The molecular basis of cancer

The Progression of a single cell from normal to a neoplastic condition always involves a sequence of genetic changes that vary either from the regulation or the function of a variety of different genes [92]. The cancer-forming process, called tumorigenesis is an interplay between genetics and the environment.The majority of cancers arise after carcinogens alter genes, or errors in copying and the repair of genes. If the genetic damage occurs in a somatic cell, a division of this cell will transmit the injury to the daughter cells, creating a clone of transformed cells [178].

Cancer does not develop suddenly. Instead, it often grows over many years with detectable premalignant lesions. Recently, significant improvement is achieved in perceiving the molecular basis of cancer as a genetic disease caused by defective genes; these genes can involve either loss or gain of gene function [131].

Tumors can separate into two main groups, benign or malignant [131]. Benign tumors are rarely life- threatening, growing in a capsule that limits their size and keeps the character of the cell, and are thus generally well differentiated. Malignant tumors invade nearby tissues and expand to different areas to produce further growth or metastases [178].

2.1.3 Cancer-Related Genes

The first clue to the identification of specific genes involved in the development of cancer comes from the study of tumor viruses. The discovery of viral oncogenes in the 1960s provided the first evidence that a particular gene could induce cancer

[26]. Cancer is caused by the genes that control cell proliferation. Two kinds of regulatory genes have been discovered. Oncogenes that promote growth and those that suppress growth are called tumor suppressor genes [140].

2.1.3.1 Proto-Oncogenes and Oncogenes

Oncogenes derive from proto-oncogenes, the normal cellular genes that provide pro-growth signals to cells. However, mutations or altered regulation of Proto-oncogenes can transform it to oncogenes [140]. The proto-oncogenes are situated in the cytoplasm or the plasma membrane and regulate the cascades of events that maintain the ordered progression through the cell cycle, cell division and differentiation [127]. Oncogenes are classified into five modules based on their functional and biochemical properties, including 1) secreted growth factors. 2) cell surface receptors. 3) components of intracellular signal transduction system. 4) DNA-binding nuclear proteins, including transcription factor. 5) elements of the network cyclins, cyclin-dependent kinase and kinase inhibitors that govern progress through the cell cycle [139]. A mutation that changes a proto-oncogene to an oncogene is dominant.

2.1.3.2 Tumor Suppressor Genes

Tumor suppressor genes normally restrain cell growth either by inhibiting the cell cycle progression or by promoting programmed cell death, their malfunction results in uncontrolled cell growth. They function in a recessive fashion at the cellular level for carcinogenesis [96]. Inactivation of one allele of the susceptibility gene is insufficient for tumor formation, alterations of both alleles are necessary for cancer development. The first mutation could be either a germline or somatic mutation, whereas the second mutation is always somatic, [191]. The first tumor suppressor gene discovered was the Rb (Retinoblastoma 1). Rb. was isolated as the first tumor

suppressor in 1987 [116] [71]. it remains a typical example of a tumor suppressor gene. Rb has additionally been shown to be involved in chromosomal instability, angiogenesis and response to hypoxia [34]. Other classical tumor suppressor genes include TP53 [25] [223], CDKN2A [173] in multiple tumor types and VHL in kidney cancer [114].

2.1.4 Breast cancer

Cancer of the breast is the most common cancer in women worldwide [56]. In the past decades, the global incidence of breast cancer has increased [29] [229] [94], accounting for 26% of all female cancers [93]. One of eight women develops breast cancer during their lifetime. Breast cancer in men is rare accounting only for 1% of all breast cancers [240]. Early cancer detection and improved treatment have lead three-quarters of the patients to survive for at least ten years. Therefore, the primary reason for cancer-associated death is not the primary tumor, but rather the development of remote metastases in other organs [11]. It is estimated that more than 1 million new cases will be diagnosed every year [160]. The past decades has seen the incidence of breast cancer increased in several parts of the world, and the disease remains the most common female cancer in developed countries [95]. The reason that contributes to the poor identification of patients diagnosed with breast cancer is the delaying in diagnosis, due to limitations of mammography. Screen-film mammography (SFM) is considered the standard for breast cancer screening and detection [152]. An available alternative to SFM includes full-field digital mammography (FFDM) that is more sensitive in women with dense breasts [157]. The major factor that contributes to breast cancer mortality is the presence of metastasis, the leading cause of death accounts for more than 400,000 deaths annually around the world [100].

2.1.4.1 Histological Subtypes of Breast Cancer

The classification of the histological types are often performed according to the world health organization (WHO) classification criteria and is grouped to: In situ carcinomas (ductal and lobular carcinoma), invasive ductal, invasive lobular carcinoma [63]. Nearly all breast cancers are adenocarcinomas, originating from the mammary glandular system. Breast cancers are classified based on tumor histopathology and the location of origin. The two most common types of breast cancer (ductal carcinoma and lobular carcinoma), both kinds are further divided into invasive, and low-invasive carcinomas. Ductal carcinoma is the most common type; it accounts for ~80% of all breast cancers while lobular carcinoma accounts for ~10% of all cases (Malhotra *et al*. 2010). An increased frequency of ductal carcinomas is seen in patients aged less than 35 years 67% compared to older patients 53% (O'Malley and Pinder, 2006). About 70-80% of ductal carcinomas express ERs, and 15-20% are HER2 positive (Ladjemi *et al*., 2010). These classification methods are used over many decades as a tool used to aid in treatment and prognosis [129].

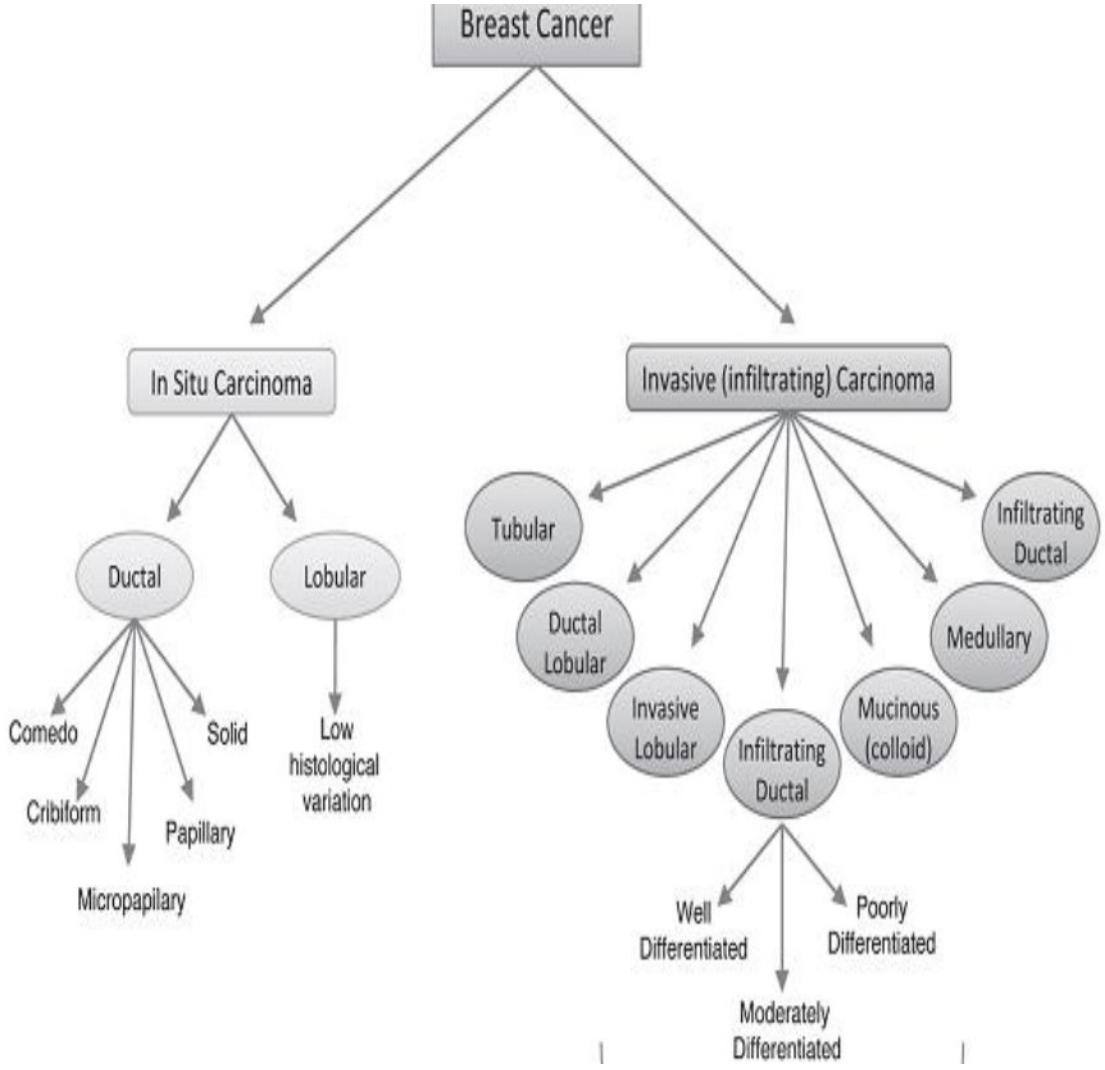

Figure 2-2: The histological classification of breast cancer subtypes [129]

2.1.4.2 Molecular Subtypes of Breast Cancer

Earlier classification of breast cancers was mainly based on tumor histology and location of origin. However, with continuing research advancement, gene expression profiling became a powerful technique to categorize breast cancers into different molecular subtypes with distinct gene expression patterns [238]. At least five different molecular subtypes of breast cancer are identified: luminal A; luminal B; HER2 enriched; basal-like and claudin-low type breast cancer [144] [151] [167]. Luminal A type tumors have the best prognosis and are low-grade tumors.

Luminal A and luminal B breast cancer subtypes make up the majority of all diagnosed breast cancer cases; both are estrogen and progesterone receptor positive that makes them respond well to endocrine therapy. However, Luminal A and B tumors differ to some extent in their gene expression pattern [129].

Recent studies identified several molecular subtypes of breast cancer that are classified to basal-like, ErbB2+. HER2-enriched breast cancer subtype expresses high levels of HER2 and is typically estrogen and progesterone receptor negative. HER2 enriched tumors make up about 12% -20% of all breast cancer cases, they are high-grade tumors and are associated with lymph node metastasis [235]. Breast tumors that lack ER, PR and HER2 are referred to as triple negative breast cancer. The basal-like tumor is a subtype of triple-negative breast cancers; they are high-grade tumors and are associated with poor prognosis [154]. More recently, a new subtype classified as "claudin-low" has been identified. Claudin-Low Subtype of Breast cancer is the most recently identified triple negative subtype; it accounts for ~ 7-14% of all breast cancer cases [85] [161]. Most of the claudin-low tumors are ductal carcinoma that rarely respond to endocrine therapy and are strongly associated with poor prognosis [161]. The knowledge of these subtypes may predict the outcome for patients clinically. Further, they may be used as predictors of clinical response to therapy [144] [167].

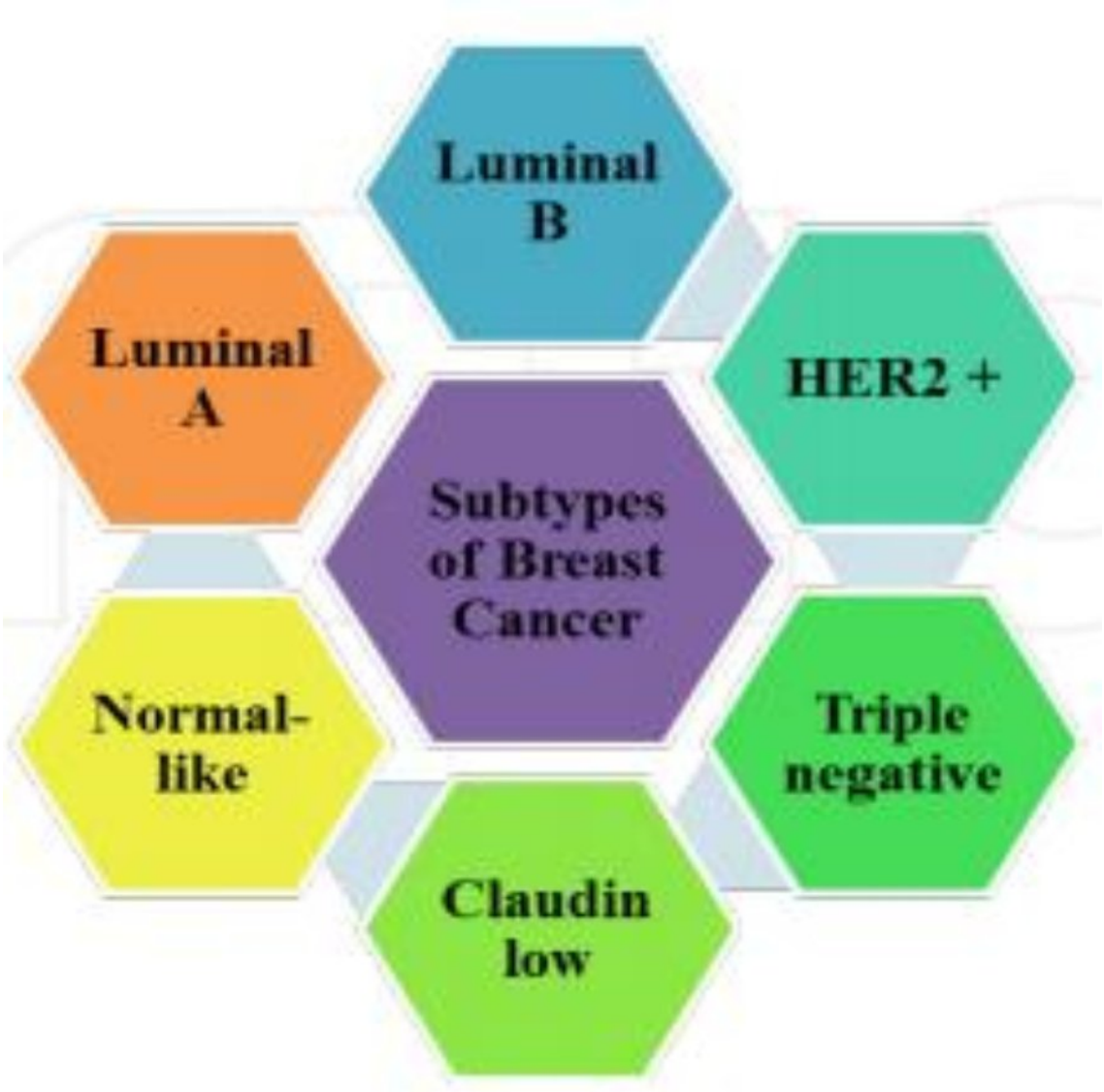

Figure 2.3 The molecular classification of breast cancer [129].

2.1.5 TNM-Classification and Staging

Management of breast cancer is based on staging and grading. TNM is an international system for staging malignancy and measures three primary parameters: T for the diameter of the tumor, N for lymph node involvement and M for the presence or absence of distant metastasis, as well as features such as tumor size. TNM classification in 2009 is the seventh modification of the classification system

[202]. The system stages breast cancer from 0-IV based on the size of the tumor, lymph node involvement, and distant metastasis. Stage 0 tumors are defined as being a precancerous tumor, Stage I refers to tumors that exhibit micro-invasion of surrounding tissues. Stage II tumors are classified based on the extent of tumor dissemination in lymph nodes. Stage III tumors are < 5cm in diameter with extensive lymph node involvement and stage IV refers to tumors with distant metastasis [196].

2.1.6 Grading

The histological evaluation of differentiation for a tumor performed according to the Bloom – Richardson method of grading [24] is based on three components; the mitotic count, nuclear pleomorphism and tubule formation [63]. The primary and typically used grading system is the three-grade scale, the differences between the three different grades are based on cellular morphology and how tumors differ from normal cells [84]. Histological grade correlates significantly with survival [125], it also highly correlated with tumor size. Tumor grade has been a highly valuable prognostic factor for breast cancer [164].

2.1.7. Diagnosis

Early breast cancer does not always cause pain, but when cancer grows, it causes changes in the breast shape or size [5] [176]. If any change appears, proper examinations should be initiated using "triple diagnosis" that includes clinical examination, mammography, ultrasonography and fine- needle aspiration for cytology or core needle biopsy for histopathology [201]. Breast cancer is often detected by clinical breast examination (CBE). Women must perform self-breast examination monthly, mostly to get familiar with the proper anatomy of the breast and be aware of abnormal changes [103]. However, the self- breast examination is

not an efficient method to detect nonpalpable masses. The first and the most useful method for the early detection is the screening program. The principal aim of mammography screening program is to detect breast cancers before cancer dissemination [227]. Annual screening, however, may be more efficient among 40-49 Years women [239] [224]. A disadvantage of mammography is that it doesn't distinguish between malignant and benign tumors, and has been shown to detect 35-45% of non-cancerous mammary calcifications [199]. Another disadvantage is that 10-20% of breast carcinomas are not detectable by mammography [75] [65]. Diagnosis of a tumor mass by clinical examination or mammography is followed by core biopsies to confirm a tumor's malignancy. Several different biopsy techniques are applied to get material of nonpalpable lesions like fine needle aspiration (FNA), and large-core needle biopsy. FNA is a well-established tool for the estimation of palpable breast lumps but it can't distinguish between invasive and in situ cancer [237]. Large core needle biopsy allows recognition of an invasive component; Additionally, it facilitates the evaluation of the tumor grade. Diagnostic accuracy of large-core needle biopsy is high 93-99% [221]. Sets of diagnostic assessments, including ultrasound tomography scan (CT) and magnetic resonance imaging (MRI), are used to examine the extent of tumor progression [21] [169]. Magnetic resonance imaging (MRI) is a screening tool for women who have a 20-25% or greater risk of breast cancer as women with a strong family history [99].

2.1.8 Treatments

There are several therapeutics for breast cancer including; surgery, radiation, chemotherapy and hormonal therapy. In recent years, many treatments have been investigated, such as hormonal and targeted therapies. Surgery is often used to eliminate tumors for early stage breast cancer [188]. Nowadays, a segmental mastectomy or breast-conserving therapy is used; this treatment maintains a regular breast appearance after the surgery [205]. Breast-conserving therapies are recommended for early stage breast cancers [69]. Adjuvant chemotherapy and radiation are also used for breast cancer management. Chemotherapy is treatment with cytotoxic drugs that have an effect on the cell division; these drugs are classified according to their mechanism of action. All effective drugs, however, have side effects [188]. Chemotherapy works by using drugs which damage DNA and kill off some of the rapidly proliferating cells [204]. The third hallmark of cancer treatment is radiotherapy, around 50 % of cancer patients are treated with radiation therapy. Studies have demonstrated that applying radiation therapy post-surgery reduces the risk of local recurrence (LR) by 50% in breast cancer patients [42]. A reduction of ~ 16% in (LR) is noticed in response to radiation therapy compared with those that are not receiving radiation [49]. Another type of treatment for breast cancer is" Adjuvant systemic therapy" which refers to the administration of chemotherapy, hormone therapy and trastuzumab (a monoclonal antibody directed against HER2). Studies have shown trastuzumab to improve the clinical outcomes of HER2 positive breast cancer treatment significantly when used in combination with chemotherapy compared with chemotherapy alone [74]. Hormone therapy benefits patients with hormone receptor-positive, excluding those with hormone receptor-negative breast cancer [22]. Hormonal therapy decreases the amount of estrogen in the body and inhibits the action of estrogen on breast cancer cells [138]. There are also molecular-specific therapeutic agents that target

specific hormone receptors [154]. Tamoxifen, a selective estrogen receptor, and an inhibitor that inhibits the growth of breast cancer cells is used for estrogen receptor positive tumors, which accounts for 70% of all breast cancers [129]. Despite survival advantages of these therapies, many breast tumors are not eradicated entirely due to many factors like acquiring resistance, significant toxicities or relapse following an initial response [246]. Genomic information can be joint with clinic pathological characteristics to generate novel diagnostic and therapeutic strategies, for the current management of this malignancy [256].

2.1.9. Breast Cancer Development and Progression

The development of cancer requires a series of genetic aberrations that initially give rise to hyperplasia followed by the appearance of abnormal cells and the development of carcinoma in situ. The non-invasive tumor mass remains restricted to the ducts and lobules of the breast and does not attack surrounding tissues; these types of tumors are called lobular carcinoma in situ or ductal carcinoma [194]. Tumors can acquire additional genetic alterations that result in progression into an invasive carcinoma, which can invade other parts of the body via lymphatics or blood circulation leading to distant metastatic, this process is referred to as metastasis [258]. There are many genes implicated in metastasis such as (matrix metalloproteinase 9 (MMP-9), vascular endothelial growth factor and Epidermal growth factor receptor EGFR [184].

2.2. Molecular Basis of Breast Cancer

Breast cancer is a heterogeneous disease and according to microarray-based gene expression profiling, can be divided into five subgroups: luminal A, luminal B, HER2-overexpressing, basal-like / triple negative and the normal like. These subtypes have different prognoses and treatment options [19] ER, PR, and HER2

status provide additional prognostic information, beyond standard histological estimation. Basal-like / triple negative breast cancers, which are ER-, PR- and HER2-negative have relatively little long-term survival rates, according to current estimates. Triple-negative / basal-like breast cancers account for 10–17% of all breast carcinomas [60] [158]. The majority of cancer cases are sporadic-appearing tumors in nature because there is no clear family history. However, some families with a known genetic cause or inherited tendency to cancer have been identified [105]. Mutations in some genes are known to cause susceptibility to breast cancer. The most significant of these are the BRCA1 and BRCA2 genes [119]. About 90 genes or genetic loci are implicated in breast cancer susceptibility [58]. Breast Cancer heritability is made up of three different classes. The first class is high-penetrance risk alleles, such as BRCA1, BRCA2, and TP53. These alleles have a strong effect on breast cancer risk. However, mutations in these genes are uncommon and therefore, only a small percentage of the population is affected [86] [73]. The second class of breast cancer risk alleles is moderate risk alleles. These usually include genes known to interact with BRCA1and BRCA2 or genes that are active in DNA repair pathways, genes in this class involve PALB2, CDH1, and CHECK2 are rare in the population [132]. The third class of breast cancer risk alleles is common, low-penetrance risk alleles that make up the majority of breast cancer heritability [73].

2.2.1 Classical Marker Genes For Breast Cancer

Markers in Breast Cancer can group into different cellular systems including 1) Cell growth markers that include; growth factor receptors, signal transduction pathway, control of the cell cycle and DNA transcription proteins. 2) Cell death markers, such as apoptosis. 3) Invasive and metastatic potential, including cell

adhesion molecules and metastasis suppressor genes. 4) Other markers for chromosome and DNA content within cancer cells [209].

2.2.1.1 BRCA1, BRCA2

BRCA1 and BRCA2 genes are two known tumor suppressor genes, which prevent cancer, if these genes were mutated and inherited from the parents, breast cancer risk would be high during lifetime [156]. BRCA1 on chromosome 17 and BRCA2 on chromosome 13 both have a role in maintaining DNA integrity. Mutations present a high risk of breast cancer with estimated penetrances of 55% for BRCA2 and 60% for BRCA1 by age 70 years [133]. The BRCA1 gene contains 24 exons. Mutations and variants are found along the entire coding region of BRCA1and within intronic sequences [68]. BRCA1 breast cancers are frequently classified as triple negative breast cancers, due to their lack of expressing ER, PR, and HER2 hormone receptor's [217]. BRCA2 is a vital component in maintaining genomic stability, and defects in its functions can cause genomic instability. Mutations in BRCA2 present breast cancer lifetime risk between 40–85% [12] [113].

2.2.1.2 TP53

TP53 was first identified in 1979 and is now well known for its frequent mutation in human tumors. Wild-type p53 protein is a transcription factor involved in the control of G1/S and G2/M phase transitions, in DNA repair, cell death as well as angiogenesis. The principal function of p53 is to induce cell death; its inactivation can produce treatment-resistant tumors, and thus, p53 status could be vital in determining tumor response to therapy [112]. 50% of cancers are related to mutated p53; scientists found that mutated p53 gene is directly involved in breast cancer, and other tumors [187]. TP53 is a tumor-suppressor gene that, after its

activation by oncogenic stress signals, promotes either cell cycle arrest, DNA repair or cell apoptosis [213].

2.2.1.3 HER2

HER2 is a tyrosine kinase receptor that is a member of the human epidermal growth factor family. The receptor is involved in proliferation and survival of epithelial cells [198]. Human (HER2) overexpression is present in approximately 15% of early invasive breast cancers, and it is an important predictive and prognostic marker [163]. Overexpression of its protein product has been found in up to 25% to 30% of human breast cancers and is associated with poorer outcomes compared to HER2 normal [136]. Trastuzumab is a humanized recombinant monoclonal antibody which targets the HER2. The use of Trastuzumab is considered as the standard of care both in early and metastatic HER2 overexpressing breast cancers [115].

2.2.1.4 Ck-19

Cytokeratin- 19 is a novel gene that is diagnosed as a specific marker for primary breast cancer, it is present in normal and cancerous epithelial cells, and seems to be the most sensitive and dependable marker in patients with breast cancer [6].

2.2.1.5 Mammaglobin -A

The human mammaglobin gene was identified in 1996 by Watson and Fleming. Mammaglobin-A is a (10kDa.) protein expressed in normal mammary gland epithelium [228]. Mammaglobin _A expression is higher in breast cancer patients than in healthy controls, indicating that it could be utilized as a marker for the diagnosis of breast cancer [57].

2.2.2 Candidate Breast Cancer Marker Gene (Osteopontin)

2.2.2.1 Osteopontin Structure

Osteopontin is a single chain of polypeptide composed of 300 amino acids [183]. It was initially characterized in 1979 from transformed mammalian cells and was also identified as transformation-specific protein in bones as a phosphorylated sialoprotein [185]. The prefix of the word indicates that the protein is expressed in bone; the suffix'Pontin is derived from ‘pons’ the Latin word for the bridge; that signifies the role of osteopontin as a linking protein. Osteopontin is an aspartic acid rich negatively charged protein; it contains an aspartate (Poly- D)-rich region, heparin, calcium binding sites and a thrombin cleavage motif [168].

Osteopontin is a member of the small integrin –binding ligand N-linked glycoprotein (SIBLING) family [210]. Cells may attach osteopontin via multiple integrin receptors (αvβ3) as well as various β1 and β5 integrin. CD44 is an additional cell surface receptor of osteopontin [39].

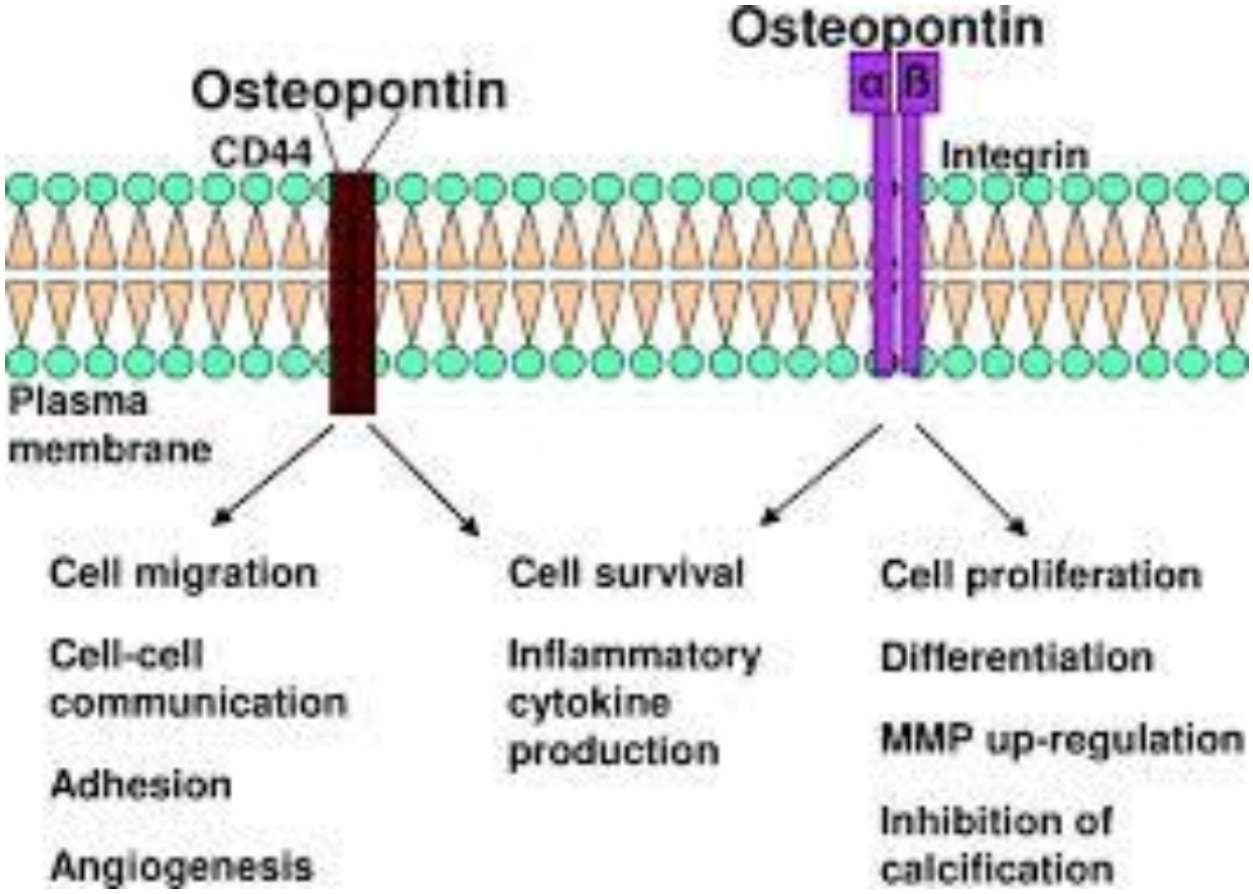

Figure 2.4 potential mechanisms whereby osteopontin may bind to extracellular matrix proteins [189].

2.2.2.2 Osteopontin gene

Osteopontin gene is also known as secreted phosphoprotein 1(spp1) 44 kDa., bone phosphoprotein, sialoprotein 1 and early T-lymphocyte activation -1-(Eta-1). The human gene for osteopontin is localized on the long arm of chromosome 4, directly related to four genes encoding for bone sialoprotein (BSP), dentin matrix protein (DMP), dentin sialo phosphoprotein (DSPP) and matrix extracellular phosphor glycoprotein MEPE [98]. Osteopontin is encoded by a single copy gene but has various isoforms as a result of alternative splicing, alternative translation, and different post-translation modifications PTMs [9]. It encoded by a single gene

with seven exons and six introns. The human gene sequence spans nearly 9 Kb. Three splice variants of the osteopontin transcript have been identified: osteopontin a, the full-length isoform, osteopontin b -lacks exon 5 and osteopontin c- lacks exon 4 [76]. Osteopontin regulates normal physiological functions such as; tissue remodeling, wound healing, bone resorption, immune response and a variety of pathophysiological conditions [3].

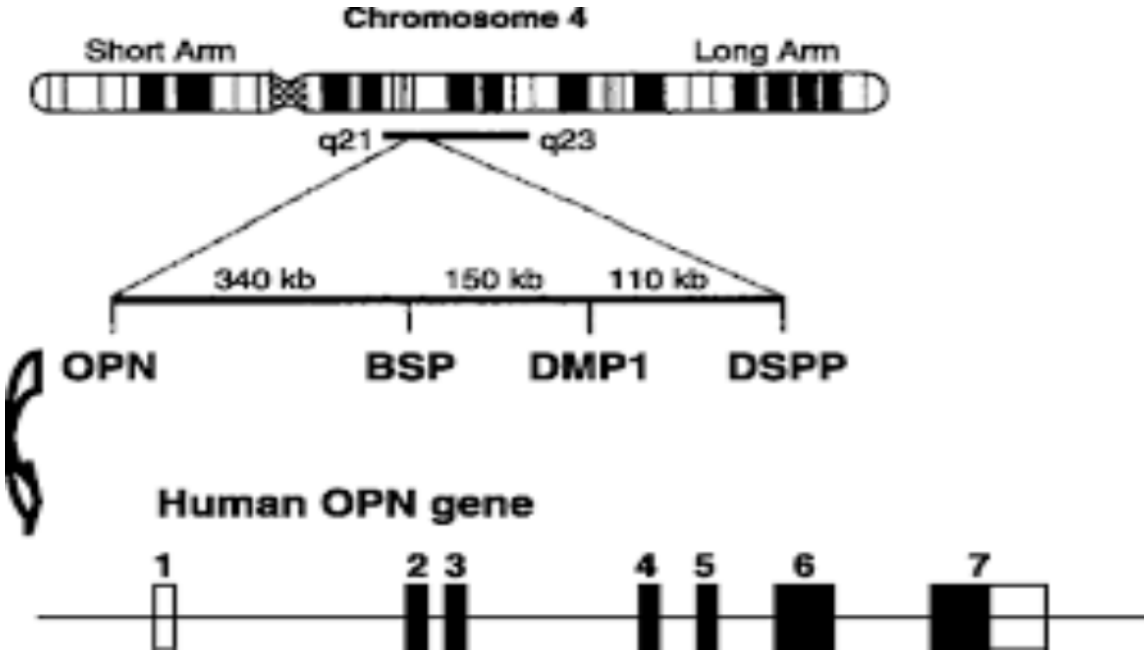

Figure 2.5 Chromosomal locations of the SIBLING genes and gene structure of human osteopontin [174].

2.3. Functions of Osteopontin

2.3.1 The role of Osteopontin in bone remodeling and wound healing

The primary function of osteopontin is the control of biomineralization with the overall negative charge; it can bind directly to specific apatite crystal faces and govern its function as a mineralization inhibitor [23]. OPN is expressed by both osteoclasts and osteoblasts, the cells that are responsible for bone remodeling [88]. Removal of bone is carried out by osteoclasts and the new bone formation by osteoblasts [97]. Osteopontin acts within bone homeostasis in three ways: promotes

the differentiation of cells to become osteoclast, inhibits the mineralization process by binding to hydroxyapatite and enhances the activity of osteoblasts [203].

2.3.2. Inflammation and Immunity:

Osteopontin is expressed in the immune system by several cell types including neutrophils, macrophages, and lymphocytes. It regulates cytokine production and cell trafficking, on the way to the vascular system, where it inhibits ectopic mineralization and macrophage accumulation [172]. It plays a crucial role in the immune response to the regulation of innate immune cells and adaptive immune cells [124]. Osteopontin mediates mucosal defense against bacterial and viral pathogens. Now it is well recognized that OPN controls immune cell functions including monocyte adhesion, migration, differentiation and phagocytosis [90] [234]. Additionally, it has also been hypothesized that OPN may play a fundamental role in the infant's immunological defense development, and it is essential for the innate immune defense in response to bacterial and viral infection [14].

2.3.3 The role of Osteopontin in tumorigenesis

OPN was discovered as a marker for transformation of the epithelial cells since 1979. From that time on, there is considerable interest in the role of OPN in human tumorigenesis [171]. It has been clinically and functionally related to cancer for many years [214]. OPN mRNA and protein are showed in histological sections of different types of human cancer to be higher compared to healthy tissue. Several studies showed OPN expression in both tumor cells and cells found in the tumor microenvironment [10]. OPN expression in tumor cells has been seen in a variety of cancer types [46]. Functionally, it changes the behavior of cancer cells both in vivo and in-vitro in nearly all cases, in a way that promotes malignancy. For

example, the addition of OPN (recombinant or transfected) to breast cancer cells in vitro, can enhance their adhesion and migration abilities, and has also been shown to alter breast cancer cells' gene expression profiles affecting genes concerned in all hallmarks of cancer [7]. OPN is overexpressed in tumors and serum of women newly diagnosed with ovarian tumors; it also elevates in the breast, prostate, lung, colon and pancreatic cancers [102]. Subsequent studies demonstrated that malignant tumors expressed significantly higher levels of OPN compared to benign and normal breast tissues [226]. The studies were further supported by other data that suggests a direct relation between OPN expression and tumorigenesis. As a result, OPN is a key oncoprotein that contributes to breast cancer invasiveness and metastasis [179].

2.3.4 The role of Osteopontin in migration and invasion

Studies have shown that osteopontin has many vital functions in cells. It was known that OPN had adhesive activity confirmed by its receptors, all mediate cell adhesion. Another well-characterized role of OPN is in regulating migration [257]. OPN mediates cell migration by acting as a soluble chemokine in the ECM and as a ligand for a variety of receptors involved in the process of migration. Several members of the integrin receptor family mediate OPN-induced migration. Integrin alphav-beta3 is most commonly associated with OPN induced migration of metastatic breast cancer cells [165]. OPN and integrin alphav-beta3 are regularly found over-expressed in metastatic breast tumors. OPN-integrin alphav-beta3 induced cell migration was also seen in several other cell types including, gastric cancer cells, and mesenchymal stem cells via some pathways [260] [123]. Moreover, it binds the CD44 surface receptor; CD44 significantly implicated in mediating cell migration, for example, OPN binding to CD44 mediates fibroblast cell migration [259]. OPN ligation to CD44 receptor has also mediated tumor cells

migration by activating the c-met kinase and has been shown to stimulate hepatocellular cancer metastasis [248]. The Protein regulates cytokine production by macrophages and has been demonstrated to act as a survival factor [52]. OPN's N-terminal fragment contains RGD motif that binds particularly to members of the integrin family. OPN's binding to integrin triggers a variety of downstream effectors that mediate cell survival and tumor growth [20]. Compelling evidence on the role of OPN in metastasis came from experiments in which transfection of tumorigenic, but not metastatic, rat epithelial cells of the breast with DNA fragments that induce OPN expression transformed the cells from benign tumorigenic cells to metastatic [18].

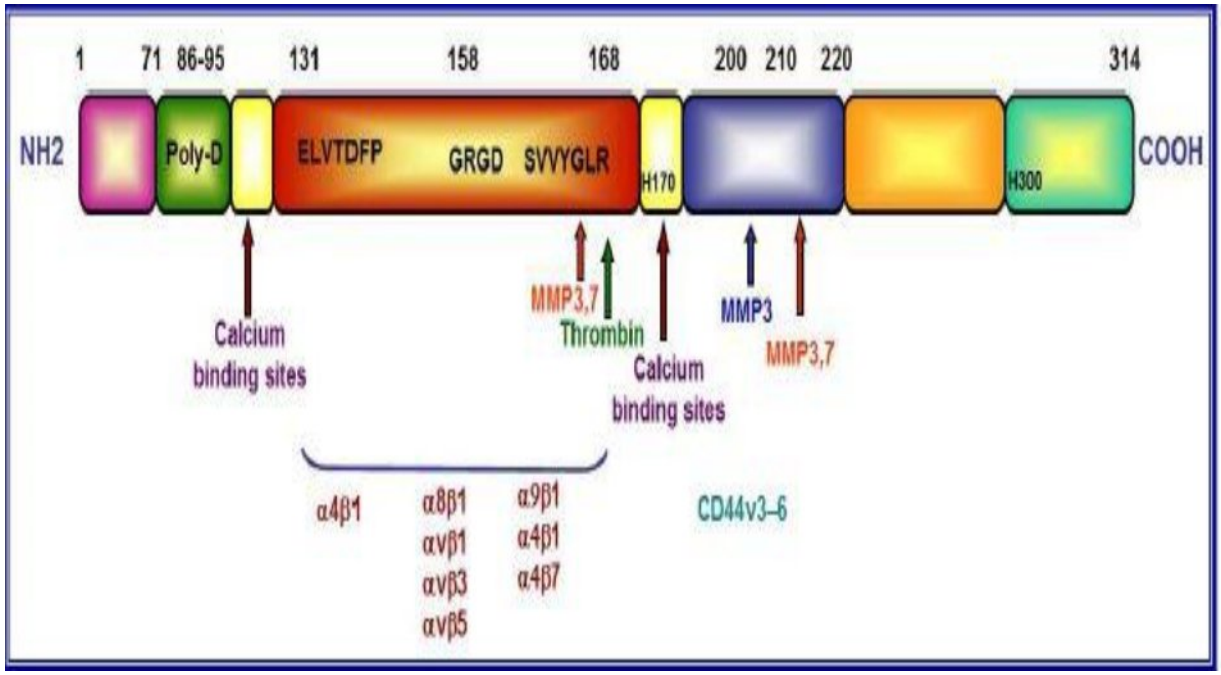

Figure 2.6 The domain structure of OPN [3].

The N-terminal contains a poly D rich.The region, calcium binding site, RGD motif, and SVVYGLR. Various integrins interact with The N-terminal domain of OPN while its C-terminal domain interacts with CD44.

2.3.5 The role of Osteopontin in angiogenesis and metastasis

Angiogenesis is a complex process which includes the formation of new vascular that is essential for sustained tumor growth and metastasis. It requires the interaction of several pro-angiogenic factors, their receptors, and adhesion molecules [37]. OPN has implications in angiogenesis by its ability to interact with integrin alphav-beta3, which is a known marker of angiogenesis [118]. Metastasis is the process by which cancer cells separate from the primary tumor and pass through the circulatory system to a distant location and form a secondary tumor [242]. OPN's role in mediating metastasis has been recognized as it can change cell adhesion properties and support cell migration, invasion and anchorage-independent survival [214], for example, transfecting mammary tumor cells with OPN lead to increasing tumor cell anchorage-independent survival. In vivo, those cells showed increased lymph vascular invasion and increased lymph node metastasis in contrast to control transfected cells [7].

2.4 Regulation of osteopontin gene expression

The expression of osteopontin is affected by some growth factors, cytokines, and hormones that can influence the rate of gene transcription, translation and post-translational modifications [51]. The regulation of OPN expression may differ between different cell types. Studies indicate that the OPN promoter region contains different motifs including a purine-rich sequence, and vitamin D response elements [106]. Transcription factors mediate OPN expression by activating response elements in the OPN promoter. OPN gene promoter contains some transcription factor response items such as the TATA-like, CCAAT- like sequences; BRCA1 and vitamin D responsive motif [230]. For example Runx2 transcription factors have been shown to induce OPN gene expression by direct binding to specific sites in the OPN promoter [59]. Other regulators of OPN gene

expression are growth factors; including transforming growth factor-beta (TGF-β), (VEGF) and (EGF). Any of these growth factors promotes OPN gene expression via a multistep signaling system [87] [117].

2.4.1 Osteopontin as a biomarker in cancer

Early diagnosis and management of effective treatment are the best strategies to fight cancer; treatment plans include monitoring cancer patients after initial treatment to detect early relapse and therapy. An approach is the stratification of patients into subgroups that respond well to different types of treatment [64]. Therefore, biomarkers are needed in many types of carcinomas. Biomarkers have improved the ability for diagnosis; prognosis and therapy prediction [107]. Nearly all of the biomarkers used nowadays have no sufficient sensitivity, specificity, or predictive value for screening. Several breast cancer markers have been studied. However, just a few proved to be useful [174]. In many publications, osteopontin has been considered one of the most promising serum and tissue marker for various cancers [3]. Osteopontin is associated with the progression of many types of cancer [231] including carcinoma of the breast [216], colon cancer [1], ovary cancer [17], prostate [70], stomach [89], lung [254] [252] ,liver [247] [121] squamous cell carcinomas [40], and sarcomas [27]. In breast cancer, high OPN expression is in plasma and tumor tissue has been associated with decreased survival [216]. Significantly, higher levels of plasma OPN is found in women with metastatic breast cancer compared to a group of healthy women. Furthermore, inside the group of metastatic cancer, patients increased OPN levels is associated with poorer prognosis and decreased survival [195]. OPN levels are also related to increased stage, grade, and larger tumor size in soft tissue sarcomas [15]. In colorectal cancer patients increased OPN mRNA is considerably correlated with stage, lymph node metastasis, and invasion [120]. The expression profiles of ER, PR, and Her2 are

used to predict the outcome and response to the therapy of breast cancer for many years. The detection of OPN-c compared to ER, PR or Her2 may be used as a diagnostic marker [108], and thus, OPN levels may be a superior biomarker to monitor disease progression and provide useful prognostic information [9].

2.4.2 Osteopontin utility as a tumor marker

Osteopontin has been detected in many human tumor types. Elevated levels of both mRNA and protein are shown in histological sections of numerous types of cancer to be elevated. Considerably higher expression levels of osteopontin mRNA are found in all tumors screened (breast, lung, colon, stomach and thyroid) as compared to related normal tissues. OPN mRNA was found to be produced by tumor-associated macrophages rather than tumor cells [33]; the Ras oncogene induces its expression, and the Ras pathway is activated directly or indirectly in a high quantity of human tumors [171]. Recently, some studies in many cancer types have sought to identify the expression of OPN isoforms if clinical and functional differences existed. It appears that OPN isoforms are differentially expressed and may have diverse effects, and this may be cancer type specific [9]. The mRNA levels of OPN splice variants a, b and c are markedly increased in glioma tissues [245] while OPN-b expression dominates in gliomas but is hardly detected in breast cancer tissues [211]. The splice variant OPN-c is uniquely expressed in breast tumors, but not in the healthy breast, in contrast, the full-length form (OPN-a) is present in both breast cancers and non-transformed breast tissues [193]. Another study, examining the mRNA from 309 breast tumors, demonstrated the role of both osteopontin and the splice variant (OPN-c) as a tumor marker for breast cancer. Quantitative RT-polymerase chain reaction was used to measure the total osteopontin and osteopontin-c mRNA levels. Results showed higher total and osteopontin-c mRNA levels in tumors of high grade, survival was significantly

shorter for patients whose tumors overexpressed the whole osteopontin, and thus, osteopontin mRNA and its splicing variant-c suggested as a marker for transformed cells [148].

2.4.3 Osteopontin as a blood marker

In addition to being present in tumors, OPN is also found in body fluids. OPN blood levels appeared to elevate in small numbers of patients with different types of cancer [186]. In 1996 Bautista *et al*. developed the first ELISA, which can quantify OPN levels in blood plasma. In 1997 Singhal *et al*. used this ELISA to quantify OPN plasma in 70 women with metastatic breast cancer compared to healthy women. OPN plasma levels were significantly elevated in women with metastatic breast cancer compared to the control group [195]. OPN serum level measured in patients with breast, lung and colon cancer was more elevated compared with normal serum levels except for colon cancer [67]. The expression of OPN-c mRNA was examined by Real-time PCR in peripheral blood from ESCC patients and healthy controls; OPN-c mRNA expression levels were higher in PBMCs of patients than in those of healthy control. The result showed that OPN-c expressed at high levels in ESCC is directly related to invasion and stage of cancer. OPN-c mRNA expression levels in PBMCs could be a potential marker for the early detection of ESCC [251]. The most likely use of blood instead of tissue biopsy for measuring changes in expression would be in osteopontin. Finally, OPN blood level can be considered as a prognostic or diagnostic marker in the breast, prostate, neck, head and other types of cancer.

2.5 Osteopontin –regulated breast cancer signaling

Osteopontin stimulates a complex of downstream signaling cascade by binding to the alpha v- beta three integrin and CD44 receptors [245]. Breast cancer

development depends on an accumulation of metastasis processes which target various signal transduction pathways. These complexes signaling mechanisms can lead to changes in gene expression which finally result in an alteration in cellular properties involved in malignancy such as migration, adhesion, invasion, angiogenesis, and metastasis [81]. OPN mediates its action by binding members of two cell surface receptor families; integrin and CD44 family. OPN-integrin alpha v-beta3 interaction mediates the activation of FAK and ERK1/2 signaling pathways which induce cellular migration [123] [260]. OPN-integrin alpha v-beta3 interaction also activates the STAT-3 signaling pathway. Signal transduction and activation of (STAT)-3 Signaling pathway is a crucial regulator of cell cycle progression, proliferation, and apoptosis [20]. OPN has also been shown to enhance VEGF expression via integrin alpha v-beta3 and mediate VEGF-induced angiogenesis [48].

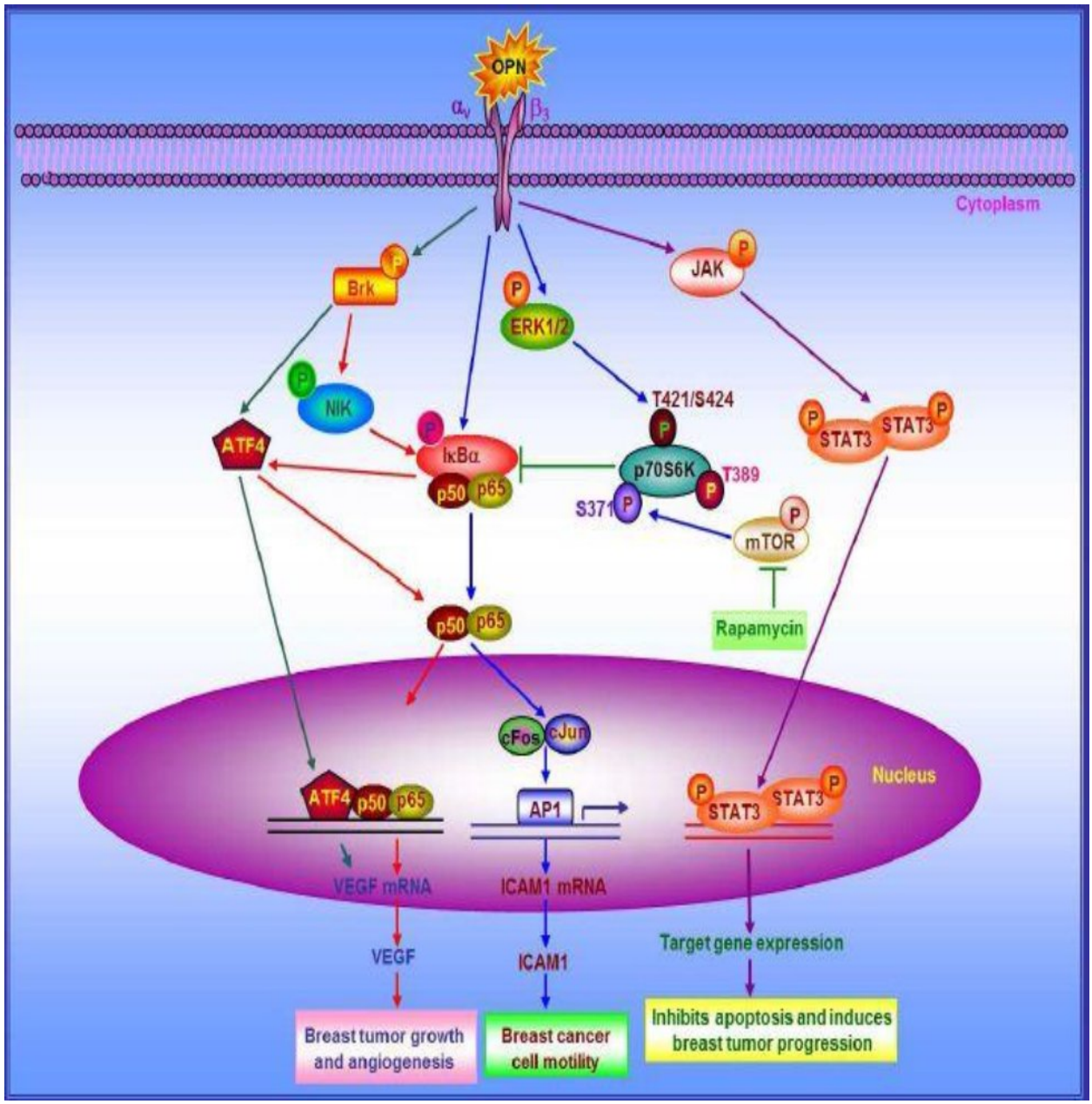

Figure 2.7 Osteopontin-induced signaling cascades mediated by its cell surface receptor, integrin [20] [2].

2.6 Osteopontin Splice variants/ Isoforms

Adding to total full-length OPN (OPN-a), there are two other splice variants OPN-b and OPN-c. The full-length OPN full length consists of 7 exons [174]. OPN-b lacks exon 5 and OPN-c, which lacks exon 4 [181] [249].

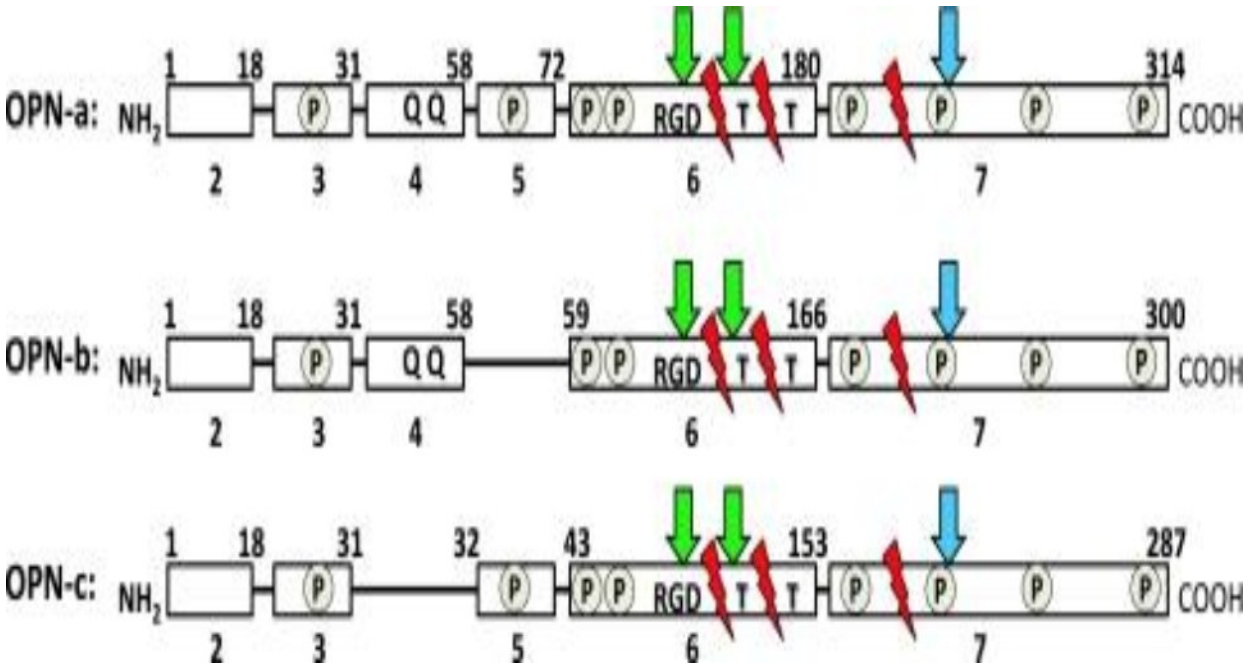

Figure 2.8 Three OPN splice variants, OPN-a, OPN-b, and OPN-c

Limited information is available on the role of the N-terminal region of OPN where alternative splicing occurs. A transformation in the splicing pattern of a protein is one of the mechanisms that can change its function and lead to malignancy-promoting effects [30]. Recently, some studies have sought to identify expression patterns of OPN isoforms in a variety of cancer types to determine whether functional differences exist [77] [91] [245]. It is shown that OPN isoforms are differentially expressed and may have various effects, and this may be cancer type specific. For instance, OPN-c is detected in breast carcinomas and invasive ductal carcinomas while healthy breast tissue was devoid of OPNc. Osteopontin-c is overexpressed in a higher grade of breast cancer [135]. High OPN-c levels are also related to tumor grade, poor prognosis and increased recurrence rates [153].

These studies recommended that OPN-c may be the most clinically significant isoform as it was found to be the primary structure expressed in tumor tissues. However, the majority of studies published on OPN and breast cancer has not differentiated between isoforms and have still recognized OPN as an active clinical prognostic factor [28] [179] [216]. Therefore, while OPN-c may indeed provide clinically valuable information, further studies must be done to clarify the appropriate assistance of the three different OPN isoforms in breast cancer. Isoform-specific studies have been done in glaucoma [245], Lung [77], pancreatic cancer [208] and mesothelioma [91]. These studies emphasize that OPN splice variants appear to have a different expression and functional property. However, it is highly dependent on cell type, and many remain to be learned about which functional roles may be variant-specific [77].

2.7 The Housekeeping Gene *(β-actin)*

The most difficult step in gene expression study is to normalize the heterogeneity of tissue samples. The ideal condition is to verify the amount of the gene transcripts by the quantity of the studied cells [104]. The levels of mRNA are in general normalized to avoid deviations such as disparities in the conditions of quantity and quality of mRNA between the samples that may be caused by pipetting errors, and differences in the effectiveness of enzymes used for reverse transcription of mRNA [16]. Presently, the most acceptable method is to normalize the data using reference genes, also known as (Housekeeping genes), which is widely used as internal controls for gene expression normalization. They are thought to be expressed in all cells of the organism at similar levels [122]. Even then, There is no single gene that could be proposed as the best housekeeping gene. Thus, the utilization of multiple housekeeping genes and checking the stability of each before analysis might yield better results. Many statistical algorithms are

developed for this purpose; Best Keeper, GeNorm, and Norm-Finder are the most popular excel based freeware software packages [35]. Optimal reference genes should stably be expressed in all tissues under investigation irrespective of tissue type, developmental stage, disease state [4]. These genes are used to normalize the mRNA levels of genes before the comparison between different specimens**.**

However, several reports have showed that variation in the expression levels of endogenous reference genes may occur as a result of pathological change mainly in clinical samples associated with malignant diseases [50] [55] [4]. The choice of appropriate housekeeping genes for clinical patient samples is vital to gene expression analysis. Suitable housekeeping genes have to be verified by data analysis for each tissue type under investigation and each experimental setup used [146]. Structural housekeeping genes involved in the cytoskeleton, like CETN2 and ACTB, demonstrated less variation than the metabolic HKGs in most tissues [177]. For the normalization of mRNA expression, the most housekeeping genes frequently used are glyceraldehyde-3-phosphatedehydrogenase (GAPDH) [62] [241], β-actin [82] and 18S ribosomal RNA [166].

Chapter Three

Materials & Methods

Chapter three

3. Material and Methods

3.1 Equipments and instruments

3.1.1. Equipment:

The list of equipments used in this work is shown in the bellow table:-

Table (3.1): Equipments:

Name	Supplied Company	Origin
Ultra-Low-Temperature Freezer -70	Sanyo	Japan
Cooling Centrifuge	Maan LAB AB	Sweden
Microcentrifuge	LabTech	Korea
Freezer -20	BEKO	Turkey
Prime Thermal Cycler	TECHNE	UK
Vortex	J.P.SELECTA,s.a.	Spain
Nanodrop	JENWAY	UK
Sensitive balance	MonoBloc	Switzerland
Ice maker	AFLO	Iraq
Autoclave	LabTech	Korea
Electrophoresis power supply	PELEX	France
Gel Electrophoresis chamber	BIOTEC-FISCHER PHERC-sub 1010-E	Germany
Multi Doc-It Digital Imaging System	AFLO	Iraq
Digital Water Bath	LabTech	Korea
Distillation	LabTech	Korea
Microwave	Gosonic	China

3.1.2 Tools

Table (3.2): Tools

Name	Supplied Company	Origin
EDTA tubes	AFCO	Jordan
PCR tubes 0.2 ml	Lab Tech	Korea
Microcentrifuge tube 1.5 ml	Lab Tech	Korea
Micropipette tips	Promega	USA
Micropipette (100 -1000)	GILSON	France
Micropipette (20 –200) µl	GILSON	France
Micropipette (10 -100) µl	GILSON	France
Micropipette (0.5-10) µl	BRAND	German
Cooling rack	Lab Tech	Korea

3.1.3 Glassware

Table (3.3): Glassware

Name	Company	Origin
Conical flask 500 ml	Bro 3.3	Germany
Graduated cylinder 100 ml	Kartell	Italy
Becker 500 ml	Bro 3.3	Germany

3.1.4 Kits and Chemicals used

Table (3.4): Kits and Chemicals used

Kits	Supplied Company	Cat. Number
Total RNA Mini Kit(Blood/Cultured Cell)	Gene aid	RB100
SV Total RNA Isolation System	Promega	Z3100
Cycle Script Reverse Transcriptase	Bioneer	E-3131 E-3132
Ultra-pure distilled water DNase-free RNase-free	Promega	P1193
GoTag® Green Master Mix	Promega	M7122
Agarose powder	Promega	V3125
TBE (10X)	Promega	V4251
DNA Marker 100 Bp. (2kb)	GeNet Bio	M-1000

3.1.5 List of Primers

Table (3.5): Primers

	Primer	Primers Sequence (5'→3')	Amplicon
*1	*B- actin gene*	F 5'-CGT GCG TGA CAT TAA GGA GA-3'	668bp
		R 5'-CAC CTT CAC CGT TCC AGT TT-3.'	
*2	*Osteopontin gene*	F 5'-TGG CCG AGG TGA TAG TGT G-3.'	148bp.
		R 5'-CGG GGA TGG CCT TGT ATG-3.'	
*3	*Osteopontin- c*	F 5'-TGA GGA AAA GCA GAA TGC TG-3'	155bp.
		R 5'-GTC AAT GGA GTC CTG GCT GT-3.'	

*Reference

- **1- [244]**
- **2- [15]**
- **3-[135]**

3.1.6 Kit Contents

3.1.6.1 Total RNA Mini Kit (Blood/Cultured Cell)

Table 3.6: Contents and quantities of total RNA Mini Kit (Blood/Cultured Cell).100 samples.

Contents	Quantities
RBC Lysis Buffer	200ml
RB Buffer	60 ml
RT Buffer	30 ml
W1 Buffer	50 ml
Wash Buffer (Add Ethanol)	25ml
RNase-Free Water	6ml
RB Column	100pcs
2ml Collection Tube	200pcs

3.1.6.2 SV Total RNA Isolation System

Table (3.7): Contents and quantities of SV Total RNA Isolation System (50 sample)

Contents	Quantities
Collection Tubes (25/pack)	2 packs
Elution Tubes (25/pack)	2 packs
Spin Column (25/pack)	2 packs
RNA Lysis Buffer(RLA)	50 ml
RNA Dilution Buffer(RDA)	20 ml
β -mercaptoethanol (48.7%)	2 ml
DNase I (lyophilized)	1 vial
$MnCl_2$,0.09M	250 µl
Yellow Core Buffer	2.5 ml
DNase Stop Solution(DSA)	5.3 ml
RNA Wash Solution(RWA)	58.8 ml
Nuclease- Free Water	13 ml

3.1.6.3. Cyclic Script Reverse Transcriptase

Table (3.8): Contents and quantities of Cyclic Script Reverse Transcriptase

Contents	Quantities
Cycle Script (200 Unit/µl)	10000 Unit
5X Reaction Buffer	0.4 ml
100 mM DTT	0.2 ml
10 mM dNTP	0.2 ml

3.2 Methods

3.2.1 Study groups

3.2.1.1 Human Blood Samples

Blood was collected from 65 sample, 55 samples were from female patients with newly diagnosed invasive ductal carcinoma, and (10) samples of healthy women as controls. All blood samples were collected in a test tube that contains anticoagulant ethylene diamine tetraacetic acid (EDTA) to prevent clotting of the blood. The blood was collected from Hiwa hospital laboratory in Sulaimani from May to September 2014, before first chemotherapy and after the first dosage of chemotherapy from the same patients. Required information about the patients and the histopathology report of the tumors were recorded from the patient's files. Healthy blood samples were provided by the Center of Early Detection of Breast Cancer in Slemani/Iraq as a control group.

3.2.1.2 Fresh tissues

Fifteen fresh breast tissues were collected from women of different ages 31-63 years old during initial biopsy performed for the diagnosis of the disease, from the Center of Early Detection of Breast Cancer. Informed consent was obtained from the individuals before the collection of samples. The diagnostic mammograms and histopathology reports revealed that out of these 15 women, 11 had breast cancer. Freshly obtained tissues were directly extracted to avoid degradation of RNA.

3.2.2 Extraction of RNA from fresh blood and tissue samples.

Total RNA Mini Kit (Blood/Cultured Cell) [222].

3.2.2.1 Fresh Human Blood

Fresh human blood was collected in anticoagulant-treated collection tubes. 1ml of RBC lysis buffer was added to a sterile 1.5 ml microcentrifuge tube (RNase-free). 300µl of human whole blood was added and mixed by inversion. The tube was incubated on ice for 10 minutes (vortex twice during incubation), then centrifuged at 4ºC for 5 minutes at 3000 × g. The supernatant was removed entirely and the cells were resuspended in 100 µl of RBC Lysis buffer by pipetting the pellet. 400 µl of RB buffer and 4µl of β-mercaptoethanol were added to the re-suspended cells, shaken vigorously and incubated at room temperature for 5 minutes. 500 µl of 70% ethanol was added and shaken vigorously. RB column was placed in a 2 ml collection tube, then 500 µl of ethanol was added; the mixture was transferred to the RB column and centrifuged at 14000 x g for one minute. The solution was discarded from the collection tube, and the remaining mixture was transferred to the same RB column, centrifuged at 14000 x g for one minute. The discarding was repeated and the RB column transferred to a

new 2 ml collection tube. 400µl of W1 buffer was added into the RB column, centrifuged at 14-16000 X g for 30 seconds, after that the flow-through was discarded and the RB column was placed back in the 2 ml collection tube. 600 µl of washing buffer was added into the RB column, centrifuged at 14-16000g for 30 seconds. The flow-through was discarded and the RB column placed back in the 2 ml collection tube, centrifuged at 14-16000 g for 3 minutes to dry the column matrix. The dried RB column was placed in a clean 1.5 ml microcentrifuge tube and 50 µl of RNase-free water was added into the center of the column matrix; left to stand for at least 1 minute and then centrifuged at 14-16000 X g for 1 minute to elute the RNA

3.2.2.2. Extraction of RNA from tissue samples (SV Total RNA Isolation System) (Size, 50 samples)

3.2.2.2.1 Preparation of Solutions

Four solutions were prepared before beginning the SV total RNA isolation system:- **DNase I**: Nuclease- free water was added in the amount indicated on the DNase I vial, gently mixed by swirling the bottle of the solution; an equal aliquots of five µl was prepared using sterile microcentrifuge tubes. Rehydrated DNase I aliquots was stored at -20º C. **RNA Lysis Buffer**: 1 ml of BME was added to 50 ml of RNA Lysis buffer, stored at 4º C, and capped tightly between uses.

RNA Wash Solution: 100 ml of 95% ethanol was added to the bottle containing 58.8 ml concentrated (RWA), stored at 22-25º C and tightly capped.

DNase Stop Solution: 8 ml of 95% ethanol was added to the bottle containing 5.3 ml concentrated (DSA).

This protocol was used for processing small tissue samples ≤ 30 mg. for best results; fresh samples were used from the system immediately after collection.

3.2.2.2.2 Procedure

1- 175µl of RNA lyses buffer (with β-mercaptoethanol added) was placed in a sterile microcentrifuge tube; the tube containing (RLA) was weighted.
2- The tissue samples were immediately lysed and homogenized in lyses buffer, and mixed thoroughly by inversion.
3- The tube containing the tissue and (RLA) was weighted, the weight obtained in step one was subtracted from the new weight.
4- 350µl of RNA dilution buffer was added to 175µl of the lysate, mixed by inverting 3-4 times and placed in a water bath at 70º for 3 minutes.
5- The mixture was centrifuged for 10 minutes at 12000 x g, and the cleared lysate transferred to a fresh tube.
6- 200µl 95% ethanol was added to the cleared lysate, mixed well by pipetting 3-4 times, and then the mixture was transferred to the spin column assembly and centrifuged at 12000 x g for one minute. The elution was discarded.
7- 600µl of RNA wash solution (RWA) was added and centrifuged for 1 minute (elution discarded).
8- DNase incubation mixture was prepared in a sterile tube by combining 40 µl yellow core buffer, five µl 0.09M $MnCl_2$ and five µl of DNase I enzyme.
9- 50µl of DNase mix was applied to the membrane for each sample and incubated at room temperature for 15 minutes.
10- 200 µl of DNase stop solution was added and centrifuged for 1 minute.
11- 600 µl RNA wash solution was added and centrifuged for 1 minute.The solution discarded from the collection tube.

12- Once more 250 µl of RNA wash solution was added and centrifuged at high speed for 2 minutes, and then the spin basket was transferred to the elution tube. Finally 100 µl of nuclease- free water was added to the membrane and centrifuged for 1 minute to elute the RNA.

3.2.3 Determination of RNA integrity

Agarose gel electrophoresis was used for the visualization of the intactness of isolated RNA directly after the extraction.

3.2.3.1 Buffers and Solutions

Agarose

RNA samples

DNA Ladder

Ethidium Bromide

TBE or TAE Buffer

3.2.3.2 Preparation of 1.5% Agarose gel

Agarose gel 1.5% was prepared as follow:

1- 1.5-gram Agarose powder was dissolved in 100 ml of 1X TBE buffer in a flask.

2- The mixture was sealed with aluminum foil, and melted in a microwave oven for (1-2) min until the agarose dissolved.

3-The mixture was removed from the microwave oven and swirled gently to resuspend any settled powder and gel pieces

4- The solution was cooled to about 50-60 ºC, and then five µl of 10mM Ethidium bromide was added.

5- Molten Agarose solution was poured into the chamber

6- Appropriate comb was set in the chamber and soaked in Agarose gel.

7- The gel was left to set for 20 minutes at room temperature, and then the comb and tape were removed carefully from the gel [182].

3.2.3.3 Loading and running the gel

1-The gel casting tray was put in the electrophoresis tank and running buffer was added.

2- 10 µl of total RNA was mixed with 2µl of loading dye and loaded onto a 1.5% agarose.

3- The gel was run at 70 V for an hour and visualized by illumination with UV light.

3.2.4. Determination of RNA yield and purity [54].

The yield and purity of RNA samples were checked using the Nanodrop which enables highly accurate analyzes of small specimens (1-2ul).

Protocol

1- The upper and lower optical surfaces of the microvolume Nanodrop were cleaned by pipetting two µl of deionized water onto the lower ocular surface. Both of the optical surfaces were cleaned with a clean, dry, lint-free lab wipe.

2- 1 µl of buffer or deionized water was added onto the lower optical surface.

3- The lever arm was lowered, and "Blank" was selected in the nucleic acid application

4- Once the blank measurement was completed, both optical surfaces were cleaned with a clean, dry, lint-free lab wipe.

5- The appropriate constant for the sample was selected A 260/A280 for RNA.

6- 2µl of RNA sample was added to the lower optical surface, and the arm was closed.

7-The measure 260/280 was chosen and all the samples were calculated. 8- RNA sample with ratio (1.7-2.1) was used directly for making cDNA. If the ratio was less than 1.7 the sample was excluded.

3.2.5 Complementary DNA(c DNA) Preparation

(Cycle Script Reverse Transcriptase)

RNA was isolated from fresh blood, and the tissue samples were reversed transcribed directly after extraction.

Procedure (20µl reaction volume)

Step one

One µg of total RNA (3-5 µl) with two µl of Oligo-dT and 2µl of nuclease-free water were added to PCR tube, mixed well by pipetting and directly denaturated for 10 minutes at 65ºC, then immediately cooled on ice.

Step two

4 µl of cyclic script reaction buffer (final concentration 5 X), 2µl of DTT (final concentration 100m M), 2µl dNTP, 1µl of cyclic script enzyme and four µl nuclease-free water were added to the mixture mentioned in step one mixed well and the reaction carried out at 42º C for 60 minutes. Finally, the tube was heated up to 95º for 5 minutes and chilled on ice. The samples were stored at -70 º C for further use.

3.2.6 Primer Design and Preparation

For amplifying a gene by PCR, primers were designed for a particular part of the gene. A primer is a strand of nucleic acid that serves as a starting point for DNA synthesis .*β-actin* gene [244] *Osteopontin* gene [15] and the splice variant *Osteopontin-c* gene [135] were ordered online and synthesized by MACRO GEN/Korea

3.2.6.1 Primer preparation 100 picomole/ µl

Preparation of all the primers were based on the synthesis of MACRO GEN

Table (3.9) *OPN-F, OPN-R* primers preparation 100 picomole/µl

Primer	***OPN-F***
SEQ	5-TGGCCGAGGTGATAGTGT-3
GC%	57.89
Tm(c)	59.5

Primer	***OPN-R***
SEQ	5-CGGGGATGGCCTTGTATG-3
GC%	61.11
Tm(c)	58.4

Table (3.10) OPN-C-F, OPN-C-R primers preparation 100 picomole/µl

Primer	***OPN-C-F***
SEQ	5-TGAGGAAAAGCAGAATGCTG-3
GC%	45.0
Tm(c)	56.4

Primer	***OPN-C-R***
SEQ	5-GTCAATGGAGTCCTGGCTGT-3
GC%	55.0
Tm(c)	60.5

Table (3.11) *β-actin –F, β-actin – R* Primers preparation 100 Picomole/µl

Primer	***B-actin-F***
SEQ	5-CGTGCGTGACATTAAGGAGA-3
GC%	50.0
Tm(c)	58.4

Primer	***B-actin-R***
SEQ	5-CACCTTCACCGTTCCAGTTT-3
GC%	50.0
Tm(c)	58.4

3.2.7 Amplification of the genes by PCR

3.2.7.1 Go Tag ® Green Master Mix

Go Tag Green Master Mix is a premixed, ready to use solution containing taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at an optimal concentration of DNA templates by PCR. Go tag green master mix contains two dyes (blue and yellow) that allow monitoring of progress during electrophoresis.

3.2.7.2 Go Tag ® Green Master Mix, 2X

Go Tag DNA polymerase is supplied in 2 X Green go taq reaction buffer, 400 µM dATP, 400µM dGTP, 400µM dTTP, 400µM dCTP and 3 Mm $MgCl_2$.
Go tag green reaction buffer is a proprietary buffer containing a compound that increases sample density with yellow and blue dyes which function as loading dyes when Agarose gel electrophoresis analyzes reaction is produced.

Table 3.12The reaction mixture (25µl) for PCR

Component	Volume	Final Concentration
2X PCR Master Mix	12.5 µl	1X
Forward primer	1 µl	0.1-1 µM
Reverse Primer	1 µl	0.1-1 µM
Template	2 µl	<250 ng
Sterilized deionized water	8.5 µl	
Total Vol. of PCR Product	25 µl	

3.2.7.3 PCR Optimization

A sample was used to carry out the gradient PCR for each primer pairs. Amplification was performed in a thermocycler. The determination of optimal annealing temperature of all the primers depended on the result of Agarose gel electrophoresis. The thermo cycler program was set to 28 cycles for *β- actin* gene and 35 cycles for both *OPN* and *OPN-C*, according to the following parameters in the table.

Table (3.13) Conditions of gradient PCR reaction

Step	Temperature	Time
Pre-denaturation	95º C	5 minutes
Denaturation	95º C	30 Seconds
Primer annealing	55ºC-60ºC	30 Seconds
Extension	72ºC	30 Seconds
Final Extension	72ºC	3 Minutes
Hold	4ºC	

The optimal annealing temperature was determined on 55º C 28 cycles for *β-actin* gene, 56º C 35 cycle for *OPN* gene, and 55ºC 35cycle for *OPN-C* gene.

3.2.8 Agarose gel electrophoresis of PCR products

1.5% Agarose and 1X TBE buffer were prepared and poured into a gel tray to the first well; 5µl of 1Kb. DNA ladder was loaded. 8µl of each PCR product was loaded into each well. The gel was run at 90 V for 60 min.,and was then visualized under UV-light.

3.2.9 Densitometric Analysis of PCR Products [218].

The reverse transcription-polymerase chain reaction (RT-PCR) has become a standard tool in gene expression analysis studies. Starting with a very small amount of material (usually total RNA), the investigator can copy the RNA by reverse transcription (RT) to produce single-stranded, complementary DNA (also known as the first-strand cDNA). The cDNA, which is much less prone to degradation than RNA, can then be amplified by PCR and quantified to determine the relative abundance of expressed genes within and between sample groups [225]. For quantifying the PCR products, a densitometric measurement was done. The photographs of the PCR gels were analyzed using ImageJ program. The intensities of the bands converted into peaks by the software. The *OPN*, *OPN-C*, and *β-actin* gene expression products were calculated from the area under these peaks. To obtain a measured value for each patient, the intensity values of the bands of *OPN, OPN-C* were divided by the intensity values of the bands of *β-Actin* gene. Thus, a ratio was obtained for each patient as shown below:

$$\text{Ratio (p)} = \frac{\text{Densitometric Intensity of } \textit{OPN, OPN-C} \text{ bands}}{\text{Densitometric Intensity of } \beta\textit{-actin} \text{ bands}}$$

The ratio values obtained for each patient were compared to detect the difference in gene expression

3.2.10 Statistical Analysis

Analysis of data was performed using (SPS) for Windows. The t-test, Kendall's tau-b, Spearman's rho, ANOVA and Host Hoc Tests were used to examine significant differences between the groups.

Chapter Four

Results & Discussions

Chapter four

Results and Discussions

4.1 Sample collection

The study was conducted on 80 samples collected in Kurdistan region / Sulaimani / Iraq; 55 of the samples were from female patients' blood with newly diagnosed invasive ductal carcinoma, ten samples from healthy women as controls and 15 tissue samples. The patients' age ranged between 31- 63 years.

4.2 RNA extraction from patients and healthy specimens.

The isolation of the RNA requires pure reagents in addition to careful preparation due to RNA sensitivity to the cleavage by nucleases. Therefore, all the reagents and materials were sterilized to avoid RNase contamination and degradation. All RNA extracted was quantified by applying NanoDrop Spectrophotometer. Absorbance reading measured at 280nm. All samples whose concentration was, at least, 100ng/µl, were included in this study. The samples were run on an Agarose gel to check for their integrity. Finally, 45 of the blood samples were included in this study with 15 tissue samples

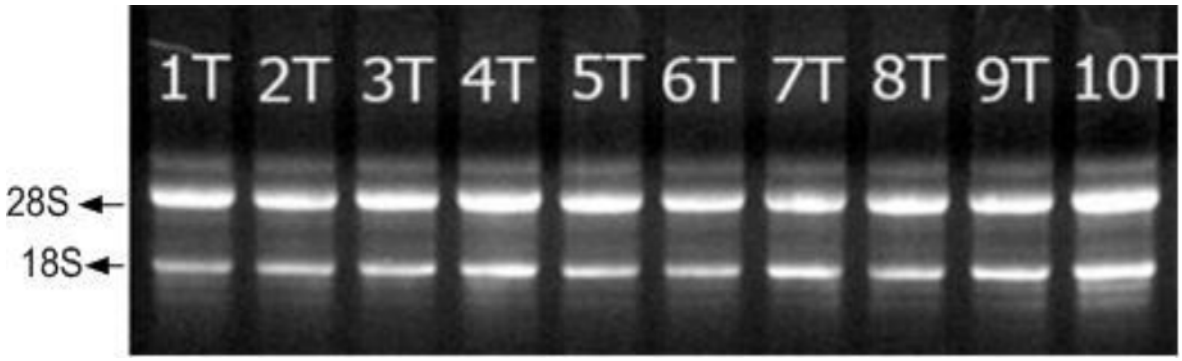

Figure 4.1 Agarose gel electrophoresis for extracted RNA from tissue samples. Agarose concentration 1.5%, lanes 1-10: Extracted RNA from tissue samples. *T: Tissue samples,

cDNA was obtained by converting RNA with reverse transcription, and then these cDNA were amplified by PCR using specific primers.

4.3 Amplification of *β-actin* gene

All samples amplified with a set of primers for the *β-actin* gene, after carrying out the gradient PCR for each primer pairs, amplification was performed. The Thermocycler program was set to 28 cycles with optimal annealing temperature 55°. *B-Actin* is a housekeeping gene that should be expressed in all samples Figures 4.2, 4.3, 4.4 and 4.5 shown the amplification of the *β-actin* gene in healthy, breast cancer patients' blood samples and breast cancer tissue samples, respectively.

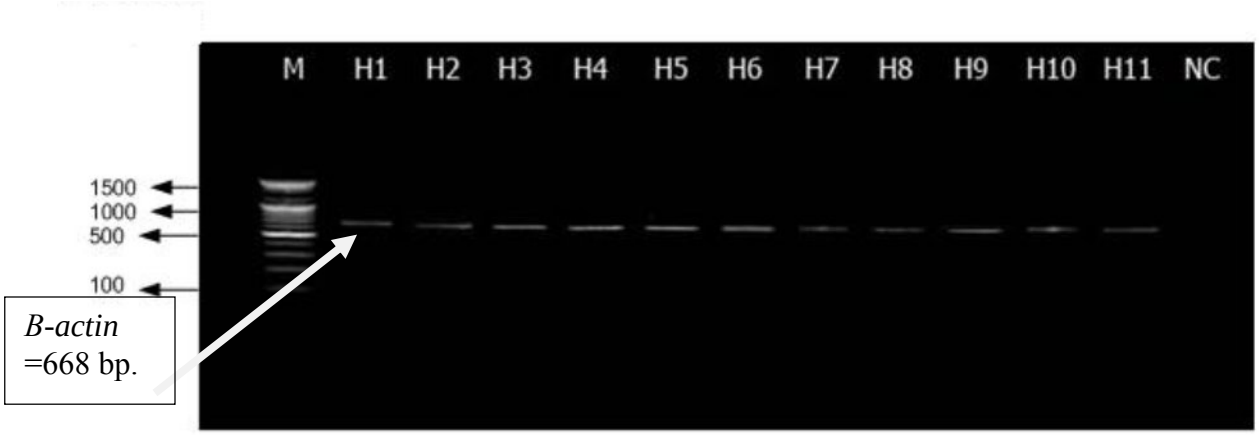

Figure 4.2 PCR products of the *β-actin* gene from healthy specimens.

Agarose gel 1.5% Lane 1: marker (2Kb DNA Ladder), lanes 2-12 *β-actin* gene from healthy blood samples. The expect product size was (668 bp.), NC: negative control. *H: Healthy samples

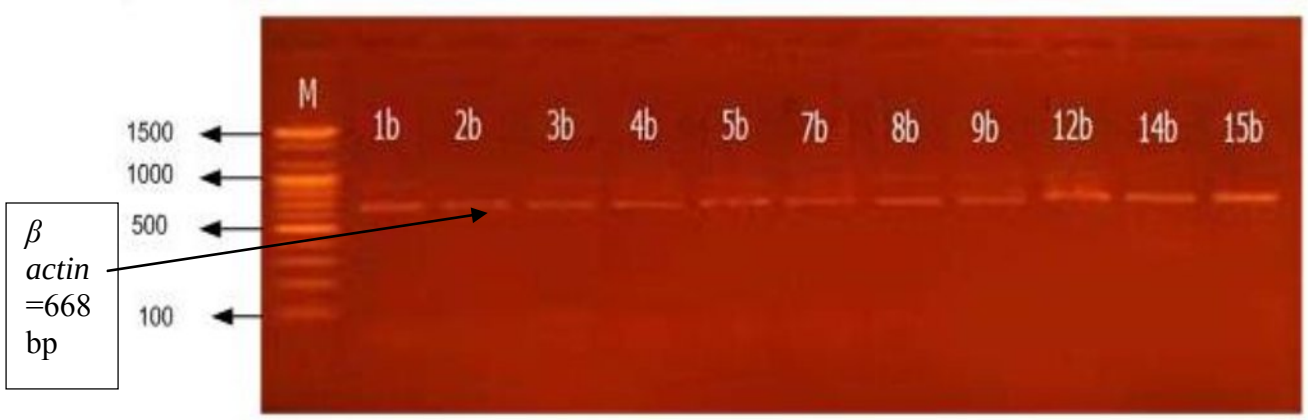

Figure 4.3 Agarose gel electrophoresis of the PCR products for a *β-actin* gene from breast cancer blood samples (before chemotherapy).

Lane 1: DNA ladder markers (2kb.), lanes 2-12: *β-actin* gene products of blood samples 1,2,3,4,5,7,8,9,12,14 and 15,before chemotherapy, the product size (668bp).*b: Before chemotherapy.

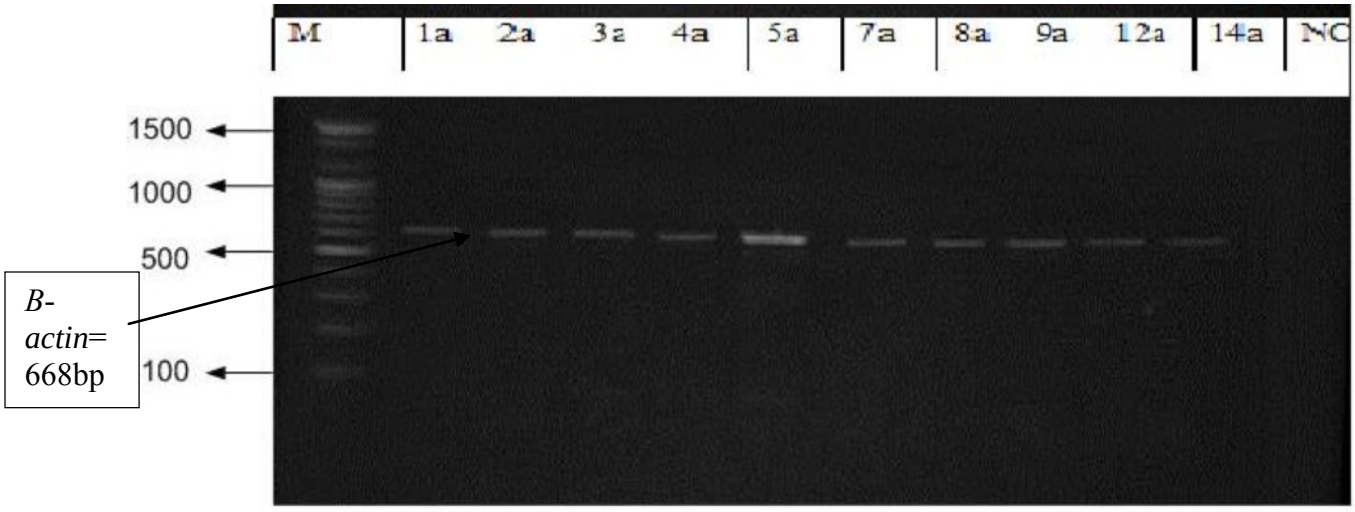

Figure 4.4 Agarose gel electrophoresis of the PCR products for a *β-actin* gene from breast cancer blood samples (After first chemotherapy).

Lane 1: DNA ladder marker (2kb), Lanes 2-11 *β-actin* gene products of blood samples 1,2,3,4,5,7,8,9,12 and 14 after first chemotherapy, product size (668bp), NC: negative control.*a = after the first dosage of chemotherapy.

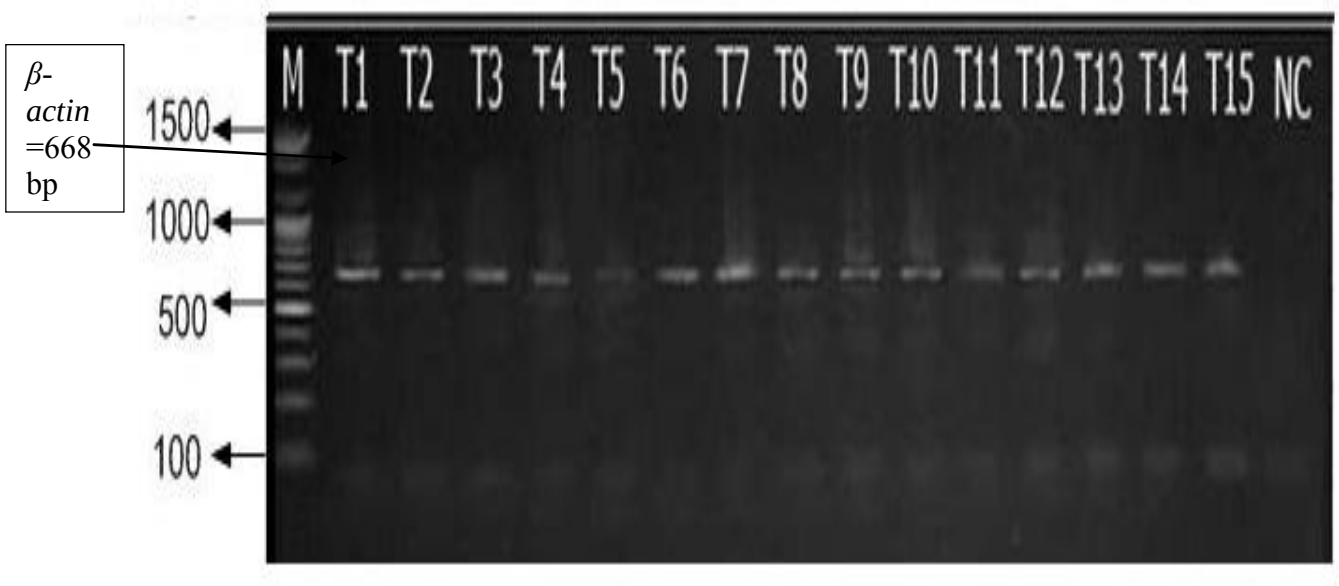

Figure 4.5 Agarose gel electrophoresis of the PCR products for a *β-actin* gene from breast cancer tissue samples.

Lane 1: DNA ladder marker (2kb), Lanes 2-16 *β-actin* gene products of breast tissue samples 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 (668bp), NC: negative control. *T = Tissue samples**.** (Agarose concentration 1.5%)

B -Actin is the most widely used gene for normalization in the experiments of gene expression [159] being a well-acknowledged loading control or housekeeping gene amplified by specific primers. From the results shown in Figures 4.2, 4.3, 4.4 and 4.5, all the bands that appeared to display the housekeeping gene *β-actin,* and it seems to be found in both the patients as well as the healthy individuals**.** The use of the internal control *β-actin* is essential to confirm the PCR conditions and normalize the variations of integrity.

4.4 Expression of *Osteopontin* (OPN) in blood samples from healthy and breast cancer patients

4.4.1 Amplification of *Osteopontin* gene in healthy and breast cancer patients' blood samples.

The amplification of *osteopontin* gene in different stages of newly diagnosed invasive ductal carcinoma, before chemotherapy, showed a band of 148bp. (Figure 4.6).

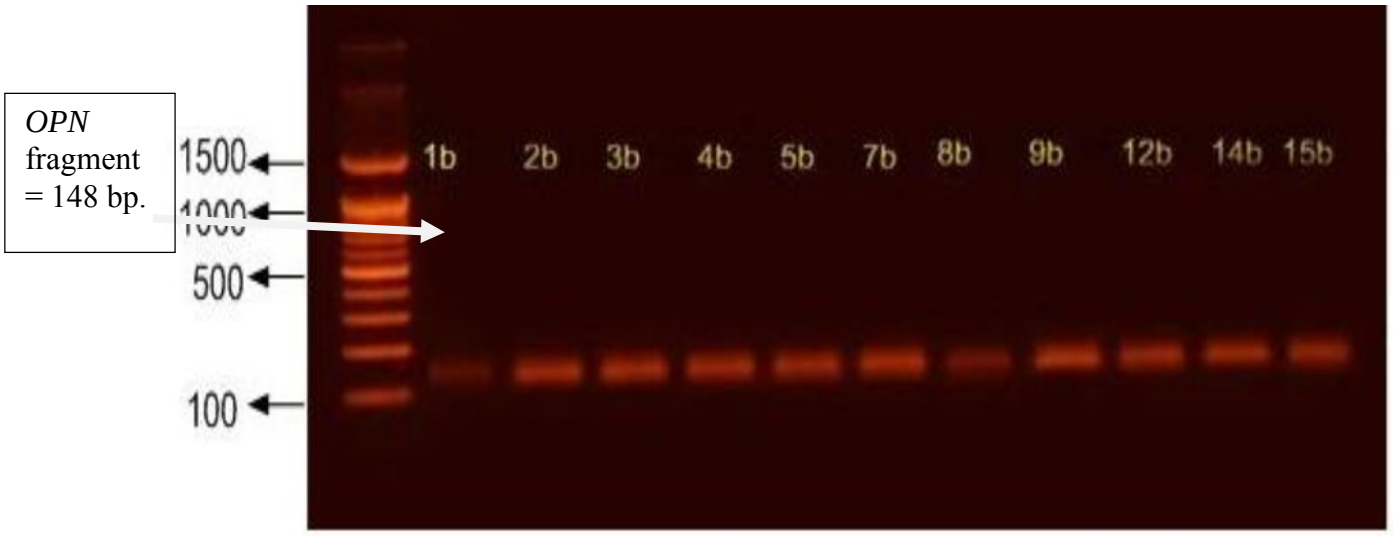

Figure 4.6 Agarose gel electrophoresis of PCR amplified product for *Osteopontin* gene before chemotherapy from breast cancer blood samples.

Lane 1: marker (2kb. DNA ladder), Lanes 2-12: PCR products of OPN (148bp) from blood samples of breast cancer patients before chemotherapy. *b = before chemotherapy (Agarose concentration 1.5 %)

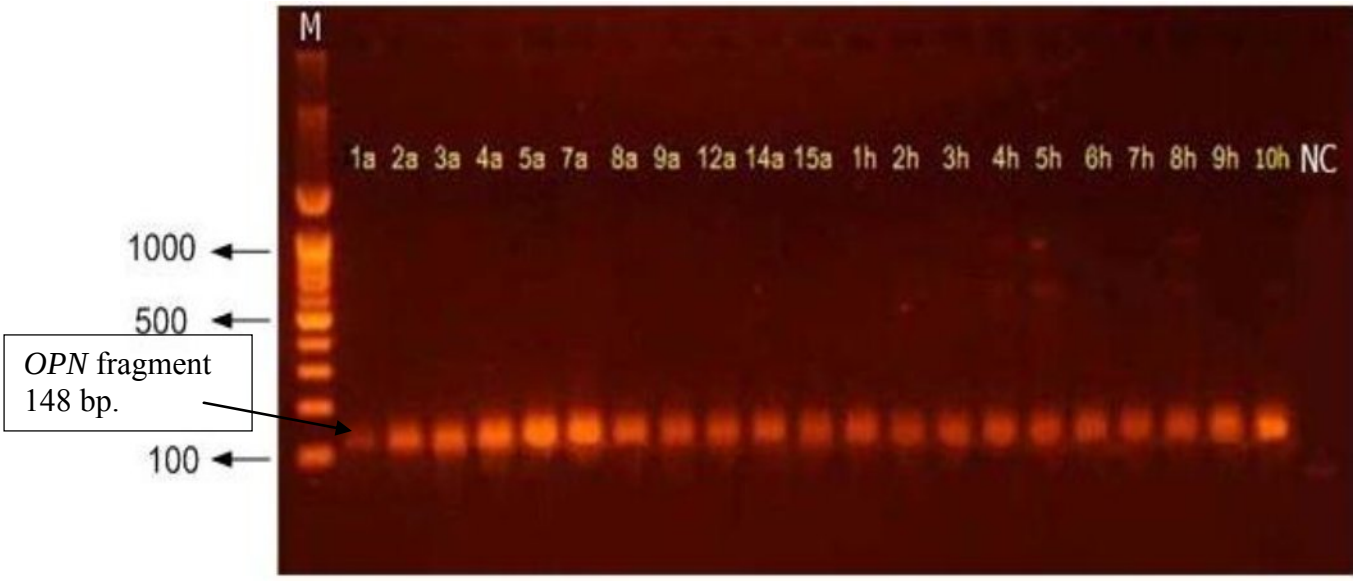

Figure 4.7 Agarose gel electrophoresis of PCR amplified product for *Osteopontin* gene after chemotherapy from breast cancer blood samples and healthy samples

Lane 1: marker (2kb. DNA ladder), Lanes 2-12 PCR products of OPN from breast cancer patients 1, 2, 3, 4, 5, 7, 8, 9, 12, 14 and 15 after the first cycle of chemotherapy (148bP). Lanes 13-22 PCR products of OPN from healthy samples (148bP), NC: Negative control. *a: after chemotherapy *h: Healthy

4.4.2 Densitometry for *OPN* gene expression in healthy and breast cancer patients:

In both healthy and patients' blood samples, the intensity of the bands was measured by image j program and the gene expression level was detected in each sample (Table 4.1, Figure 4.8).

Table 4.1 Results of Densitometric analysis for *OPN* gene expression in healthy and breast cancer patients blood samples

Sample No.	Healthy OPN/β-actin	Breast cancer patients OPN/β-actin
1	**0.7**	**0.9**
2	**0.5**	**2.8**
3	**0.4**	**2.9**
4	**0.4**	**2.5**
5	**0.4**	**2.1**
6	**0.4**	**2.1**
7	**0.7**	**1.1**
8	**0.7**	**1.9**
9	**0.6**	**1.1**
10	**0.8**	**1**

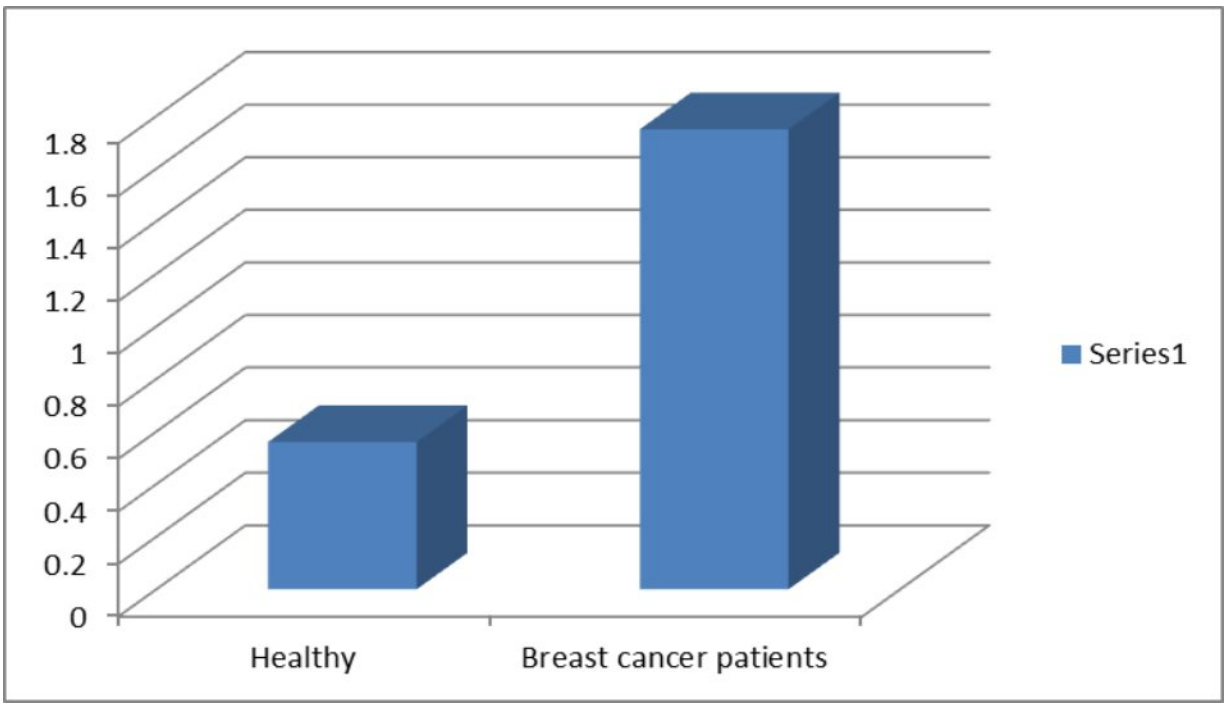

Figure 4.8 Comparison of the mean OPN gene expression between healthy and breast cancer patients before chemotherapy.

Molecular techniques are promising tools that may deal with the major problems of breast cancer detection [101]. The search for molecular markers that can be used for the early diagnosis of cancer has become one of the most significant objectives of clinical research [134]. According to its elevated expression in various types of cancer, OPN appears to have a crucial role in the process of tumorigenesis [67]. The results of *OPN* gene expression analysis in the groups analyzed (Table 4.1) showed that OPN expressed in both healthy and patient's blood samples. OPN detected in all body fluids including blood as mentioned and this is in agreement with [186] [207]. The purpose of using blood is that blood samples are easily available and can be collected at a low cost making them an attractive alternative modality for diagnostic purposes [15]. Another reason for using blood cells as a way to monitor a malignant disease in the body based on the hypothesis that malignant growth causes typical changes in

the environment of blood. These changes will influence the expression pattern of individual genes in blood cells [190].

The current study assessed the OPN gene expression levels in both healthy women, and women with breast cancer. Patients with newly diagnosed invasive ductal carcinoma (Table 4.1, Figure 4.8) revealed elevated levels of OPN in their blood compared to the standard control group. It was noticed that there was a significant difference between the levels of osteopontin mRNA expression in the blood of breast cancer patients compared to healthy samples ($P \leq 0.05$). This result matches with results of [186] which detected elevated levels of OPN in blood by western blotting. It also matches with the results of [195] who showed high levels of OPN in plasma of 70 women with breast cancer compared to healthy women. Another result by [253] reported the elevated level of OPN-c mRNA in blood of esophageal squamous cell carcinoma patients compared to healthy control and was closely related to invasion and stage of cancer. Taken together, this list of studies suggests that expression levels of OPN have a potential use as a diagnostic marker, and thus blood can be used to develop a gene-expression-based test for early detection of breast cancer.

By using the same previous method (Densitometric analysis), OPN gene expression level was measured before and after the first cycle of chemotherapy for the blood samples collected from breast cancer patients (Table 4.2, Figure 4.9).

Table 4.2 Results of Densitometric analysis for *OPN* gene expressing before and after the first cycle of chemotherapy.

	Before Chemotherapy			After Chemotherapy		
Patient No.	OPN	β –actin	OPN / β-actin	OPN	β - actin	OPN/β-actin
p.1	4975	5588	**0.9**	2699	4183	**0.6**
p.2	12935	4655	**2.8**	4723	5186	**0.9**
p.3	13751	4756	**2.9**	5828	6264	**0.9**
p.4	14656	5790	**2.5**	7166	6527	**1.1**
p.5	14128	6636	**2.1**	8039	12267	**0.7**
p.6	15026	7077	**2.1**	7307	8492	**0.9**
p.7	7856	7014	**1.1**	5310	5143	**1**
p.8	14503	7788	**1.9**	4594	5744	**0.8**
p.9	12118	11306	**1.1**	4165	3917	**1**
p.10	11478	10957	**1**	4530	3795	**1.2**

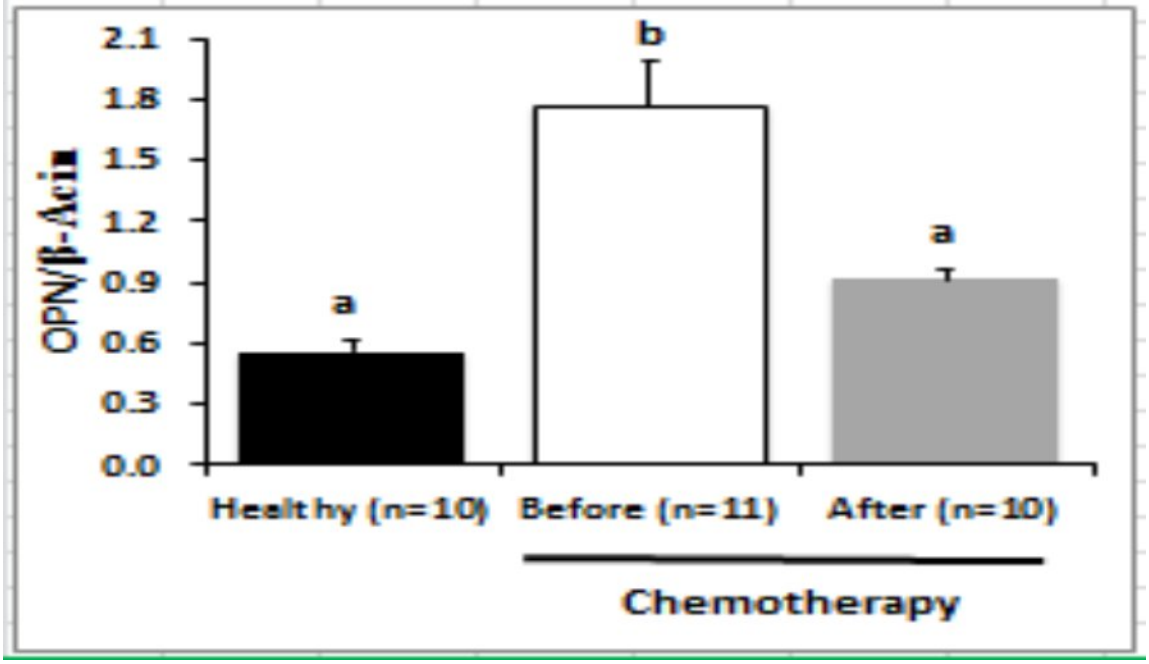

Figure 4.9 Comparison of the average OPN gene expression among healthy, breast cancer patients' blood samples, before and after the first cycle of chemotherapy.

Cancer patients' resistance to chemotherapeutic drugs complicates the treatment procedure applied and end into bad results regarding the health conditions of the treated patient. Among different types of cancer disease, breast cancer is thought to be moderately responsive to chemotherapy. The extracellular matrix protein Osteopontin plays multiple roles in the proliferation and metastasis of cancer cells [141]. The results of *OPN* gene expression both before and after the first cycle of chemotherapy are shown in (Table 4.2, figure 4.9). Before chemotherapy, *OPN* gene expression was significantly elevated compared to the values obtained after the first cycle of chemotherapy ($P \leq 0.05$). In 90% (9/10) of 10 patients, the mean value of the expression levels before chemotherapy was 1.7 whereas this value decreased to 0.9 after the first cycle of chemotherapy. The relationship between clinical response to chemotherapy and OPN has been studied

previously in lung cancer [128] where it was reported that in chemotherapy-treated patients with advanced NSCLC, the overall *OPN* gene expression was found to be related to clinical drug response of the treated patient. Thus, *OPN* levels may be a good biomarker to monitor disease progression, in addition to providing useful prognostic information.

This study has some limitations, the relatively short duration available for sample collection had an impact on the available samples especially those for post-treatment and follow-up period during the cycles of chemotherapy. Previously [8], it was shown that during the patient's treatment when the patient's get additional cycles of chemotherapy, a statistically significant increase in OPN expression levels was detected at later cycles (5-7) compared to the ones in earlier cycles. The increase in Plasma OPN levels as a result of chemotherapy occurred for most of the treated patients. Thus, serial measurements may be of limited importance in determining the response of breast cancer patients to chemotherapy. Also, cytotoxic chemotherapies used for cancer treatment can stimulate the immune system with many possible immune-regulatory effects, including a significant increase in OPN plasma levels observed in patients over the course of chemotherapy, which are from two different sources: *OPN* expressed by immune cells as a part of the inflammatory response, and that secreted by the primary tumor itself.

Further study is required to determine the accurate source of plasma OPN detected during treatment, and which mechanisms and pathways involved in it [8]. Thus, using blood samples for the OPN expression detection is unlikely to serve as a particular cancer blood marker till now, and that is because *osteopontin* blood levels elevate during kidney as well as cardiovascular disease[171]. Apparently, more trials are needed to evaluate the ability of OPN blood levels to provide prognostic information and indication of treatment response.

Following the identification of *OPN* in blood, biopsies were performed for more accurate diagnosis of the disease.

4.4.3 Osteopontin gene expression in breast cancer tissue samples.

All the *OPN* cDNA from tissue samples were subjected to sq-RT-PCR. The results revealed the presence of 148bp. Amplified product on Agarose gels electrophoresis (Figure 4.10). The densitometric analysis used for measuring OPN gene expression level for all tumor grades (Table 4.3).

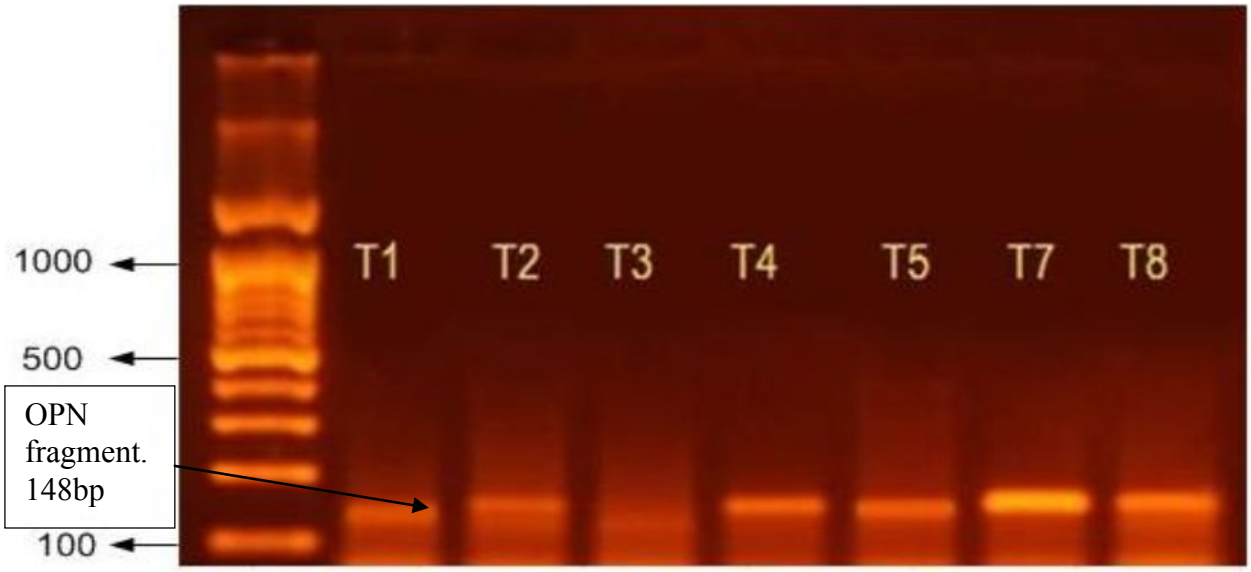

Figure 4.10 Agarose gel electrophoresis of PCR amplification product for *Osteopontin* gene from tissue samples.

Lane 1: marker (2kb.DNA ladder). Lanes 2, 3, 5 and 6 (invasive carcinoma grade II), lane 4 (Healthy), lane 8 (Invasive carcinoma Grade I), lane 7 (Invasive carcinoma Grade III) *T: Tissue (Agarose concentration 1.5%)

Table 4.3 Densitometric analysis of *OPN* gene expression from tissue samples in correlation with the tumor grade.

Sample No.	Tumor Grade	*OPN*	*β - actin*	*OPN/β-actin*
T1	Grade II	12192	5383	**2.3**
T2	Grade II	14578	3920	**3.7**
T.3	Healthy	9865	5847	**1.7**
T.4	Grade II	15130	4663	**3.2**
T.5	Grade II	15677	4722	**3.3**
T.7	Grade III	24745	8871	**2.8**
T.8	Grade I	20950	5477	**3.8**

OPN mRNA existed in both breast cancer tissues and healthy tissues (as showed in (figure 4.10) with elevated levels in the samples obtained from patients' tissues, compared to the healthy females examined. The result was in agreement with previous results [171] that reported both *OPN* mRNA and protein levels in histological sections of several types of human cancer disease are more elevated compared to the levels obtained from tissue of healthy females. Related to the results of the current study [195] reported the existence of a correlation between the high *OPN* levels related to breast cancer disease in both plasma and tumors' of the patients. The first demonstration of *OPN* expression associated with breast cancer was in a study that compared different human tumors to corresponding normal tissues [33]. *OPN* expressed in several tissues in the human body, also expressed in increased levels by tumor cells from multiple cancer types [192] with OPN expression levels being significantly higher in malignant tumors compared to those in the normal breast [179] [226]. Functionally, OPN alters the behavior of cancer cells in a manner that promotes malignancy and has also shown to change the breast cancer cells expression, affecting genes involved in all six hallmarks of

a cancer disease [45] [7]. Furthermore, studies showed a significant elevation in the levels of OPN in patients with metastatic breast cancer [28] in addition to other types of cancer invasiveness and metastatic [215] [38] [232].

As shown in (Table 4.3) there was no correlation in mRNA levels quantified by RT-PCR with tumor grade; this is in agreement with a previous study [226] that reported no differences in RNA levels quantified by RT-PCR when comparing tumors with samples from healthy individuals. Similar results reported by [216] who showed that protein levels have the potential to provide more useful prognostic information than RNA levels quantified by tumor tissue, at least for breast cancer. In contrast, a study conducted by [15] reported that *OPN* levels are associated with increased stage and grade in soft tissue sarcomas. Moreover, the findings of [120] showed that increases in *OPN* mRNA levels correlate with stage, lymph node metastases in colorectal cancer. Based on the results obtained, the current study assumes that the measurement of *OPN* in the blood or tumors of patients with breast cancer may provide valuable diagnostic and prognostic information about breast cancer disease development. However, this association was not verified due to the limited sample size and duration of the study.

4.5 *Osteopontin-c* expression in healthy and breast cancer patients

4.5.1 *Osteopontin-c* amplification in blood samples

The Thermocycler program was set to 55°for 35 cycles after adjusting the optimal annealing temperature by gradient PCR. The results of PCR for *OPN-c* gene expression for breast cancer blood samples showed the presence of (155bp) bands on Agarose gel electrophoresis (Figure 4.11, 4.12). Image j program measured the intensity of the obtained bands, and the gene expression level was detected in each sample as shown in Table 4.4.

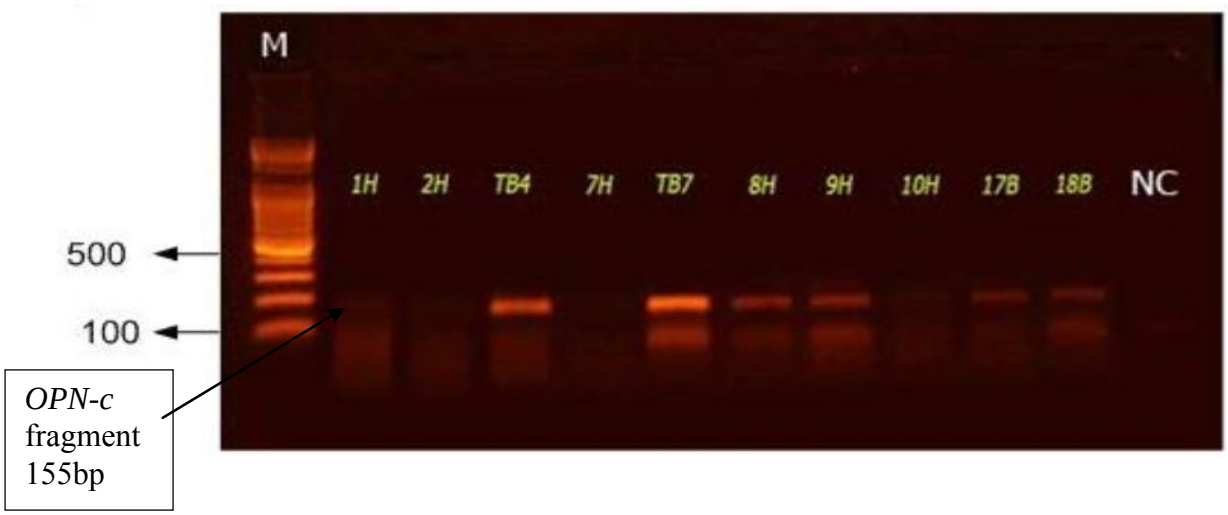

Figure 4.11 Agarose gel electrophoresis of PCR products for the *osteopontin-c* gene in blood samples.

Lane 1: marker (2kb.DNA ladder). Lanes 2, 3, 5 and 9 (healthy). Lanes 7 and 8 *OPNc* products from women with breast inflammation. Lanes 4, 6, 10 and 11 (155bp) *OPN*-c gene from breast cancer patients blood of different stages that were newly diagnosed; NC: negative control. *H: healthy, TB: blood of breast cancer patients, B: before chemotherapy**.**

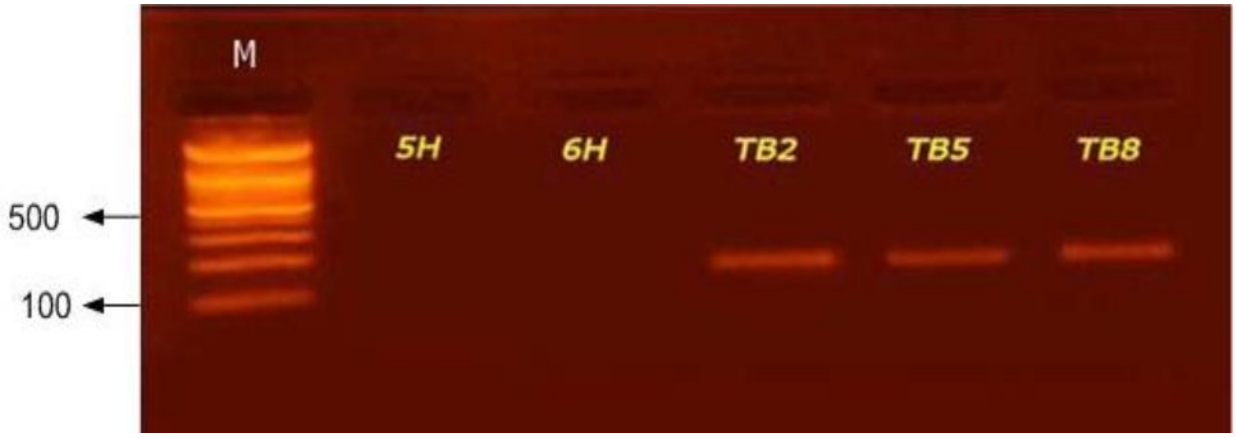

Figure 4.12 Agarose gel electrophoresis of PCR products for the *osteopontin-c* gene in blood samples.

Lane 1: marker (DNA ladder 2kb). Lanes 2 and 3 (healthy) Lanes 4, 5 and 6 (155bp) *OPN-c* from breast cancer patients *H: healthy, TB: blood of breast cancer patients

Table 4.4 Results of Densitometric analysis for *osteopontin-c* mRNA expression in blood samples

Patient	***OPN-c***	***β-actin***	***OPN-c/β-actin***
H1	3561	3778	**0.9**
H2	1805	1914	**0.9**
H5	79	2812	**0.02**
H6	241	4033	**0.05**
H7	1274	3318	**0.4**
H8	12718	7564	**1.7**
H9	14065	6146	**2.3**
H10	3176	8178	**0.4**
b17	7091	6861	**1.03**
b18	7561	6894	**1.1**
TB2	25425	8189	**3.1**
TB4	23958	8092	**3**
TB5	17125	5666	**3**
TB7	35200	6178	**5.7**
TB8	19690	6049	**3.2**

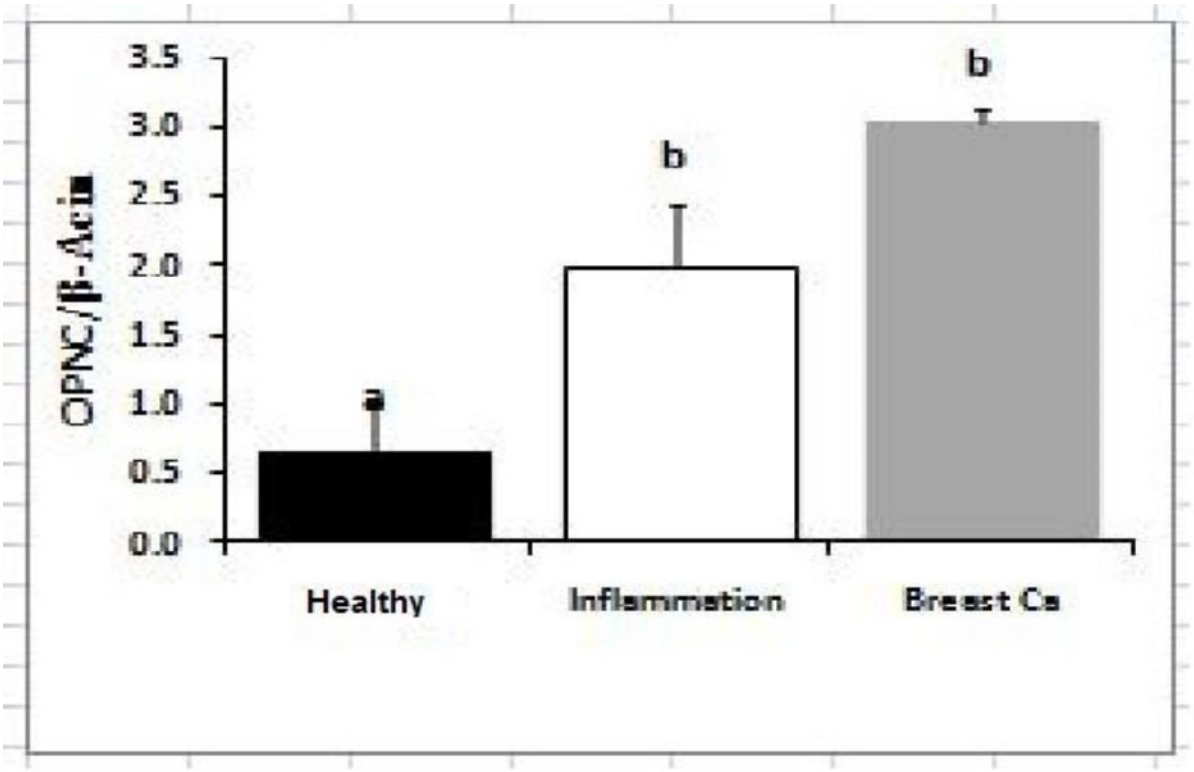

Figure 4.13 Comparison of *osteopontin-c* gene expression in blood samples from healthy women, women with inflammation in the breast and women with breast cancer.

Table 4.5 Results of Densitometric analysis for OPN-c gene expression in the blood of healthy women, women with breast inflammation and women with breast cancer.

Patient No.	Healthy	Inflammation	Breast cancer
H2	0.9		
H7	0.4		
H9		2.3	
H8		1.7	
TB2			3.1
TB4			3

Although *Osteopontin* is identified as a marker for cancer progression, the elevated production of this protein is not specific for cancer. The current study determined that the splice variant Osteopontin-c (OPN-c) is absent from healthy tissues and confirmed that it can associate with breast cancer development as suggested previously [250]. For this reason, Osteopontin splicing variant-c was proposed as a marker for breast cancer [148].

The present study examined the levels of *OPN-c* mRNA in both blood and tissue from breast cancer patients and healthy control using sq-RT-PCR technique; this technique is a powerful and rapid tool for the determination of *OPN-c* mRNA levels as a consequence of cancer disease enhancement and the estimation of the potential *OPN-c* diagnostic and prognostic values. Samples analyzed in figure (4.11, 4.12) showed that *OPN-c* expressed in breast cancer blood samples with high expression levels, and the levels decreased in women with inflammation in the breast (samples H8 and H9), also in breast cancer patients after surgery (b17 and b18) as shown in table (4.4) but not expressed in healthy blood samples tables (4.4 and 4.5) which are similar to the results obtained by [80]. As shown in figure (4.13) there is a significant increase in *OPN-c* mRNA expression levels in breast cancer samples compared to healthy specimens ($p = 0.011$) which mean there is a correlation between OPN-c levels and breast cancer. The result is in agreement with a study conducted by [233] who showed that breast cancer is associated with higher OPN-c blood RNA than in situ carcinomas and healthy. Also [231] reported that osteopontin splice variants mRNA blood level may use for monitoring cancer progression.

4.5.2 Amplification of osteopontin-c gene in tissue samples

Fifteen tissue samples were collected from women during initial biopsy performed for the diagnosis of the disease and then subjected to sq-RT-PCR for

the purpose of determining the expression level of *OPN-c* gene. The results showed (155bp) of the amplified product (Figure 4.14, 4.15) (Table 4.6).

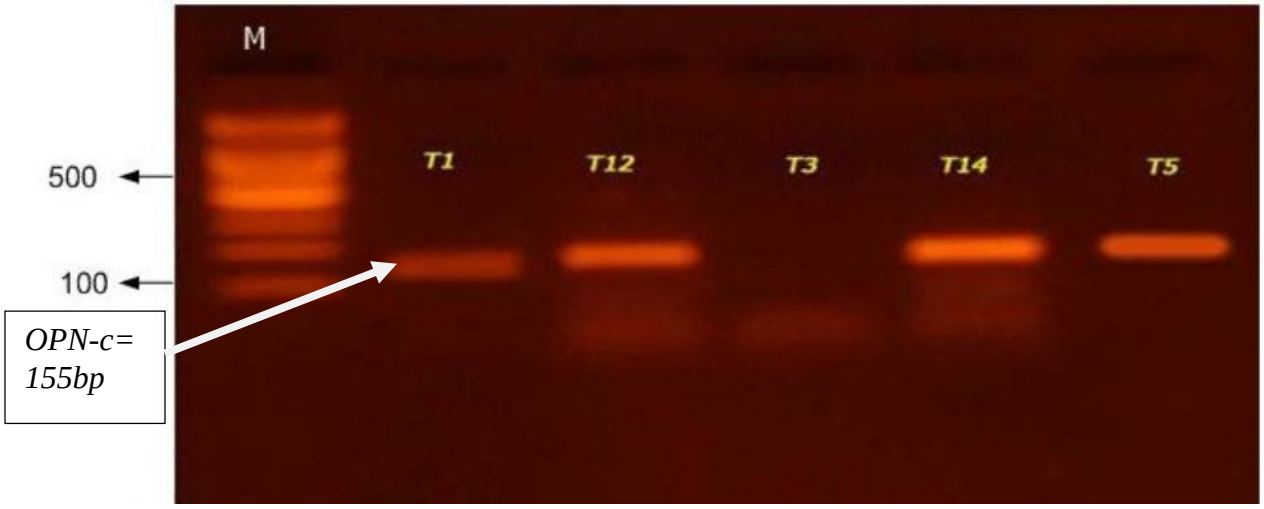

Figure 4.14 Agarose gel electrophoresis of PCR amplification product for *Osteopontin c* gene from tissue samples.

Lane 1: marker (DNA ladder 2kb) Lanes 2, 3, 5 and six amplification product of *OPN-c* in breast cancer tissue samples (155bp). Lane 4: OPN-c in a healthy specimen.

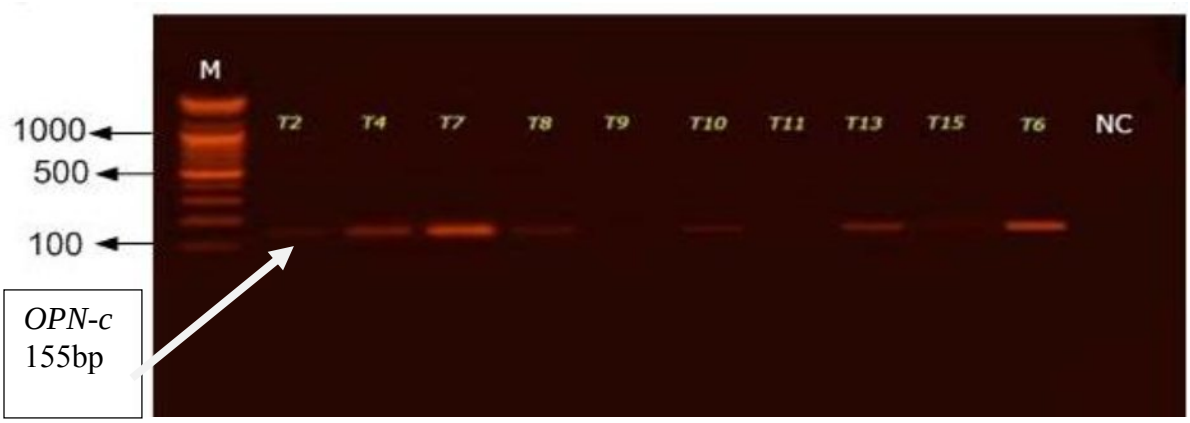

Figure 4.15 Agarose gel electrophoresis of PCR amplification product for *Osteopontin c* gene from tissue samples.

Lane 1: marker (DNA ladder 2kb) Lanes 2, 3, 4, 5,7 and 9 (155bp) *OPN-C* from breast cancer tissue samples. Lanes 6, 8 and 10 OPN-C from healthy tissue samples, NC: negative control

Table 4.6 Results of the Densitometric analysis for OPN-c expression from human tissue samples

Patient No.	***OPN-c***	***B-actin***	***OPN-c/β-actin***
T1	13345	5383	**2.5**
T2	5015	3920	**1.3**
T3	794	5847	**0.1**
T4	17413	4663	**3.7**
T5	18983	4722	**4**
T6	21211	5803	**3.7**
T7	36360	8871	**4.1**
T8	8683	5477	**1.6**
T9	3265	6899	**0.5**
T10	9392	6000	**1.6**
T11	1998	7003	**0.3**
T12	24843	7145	**3.5**
T13	17891	6665	**2.7**
T14	30879	6966	**4.4**
T15	7230	5377	**1.3**

The table shows that OPN-c in breast cancer patients expressed in elevated mRNA levels compared to the corresponding levels expressed in the healthy tissues. The expression of OPN-c in breast cancers, but not in healthy control samples may provide a simple reliable diagnostic and prognostic marker.

Table 4.7 Results of Densitometric analysis comparing between *OPN-c* gene expressions in different grades of breast cancer patients.

Patient No.	Grade I	Grade II	Grade III
T8	1.6		
T10	1.6		
T1		2.5	
T12		3.5	
T5			4
T7			4.1

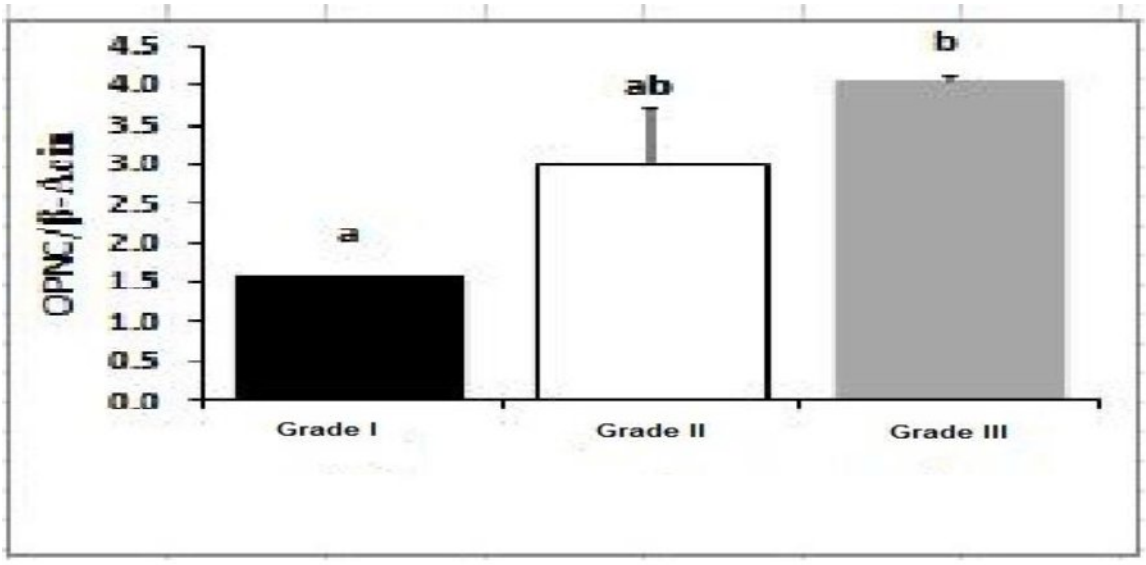

Figure 4.16 Analysis of osteopontin-c expression levels in different grades of breast cancer.

OPN-c is expressed in breast cancer tumor tissues (figure 4.14 and 4.15 respectively) but not in normal tissues. The result was similar to the results of [81] [208] as both research groups reported the presence of osteopontin-c during different cancer diseases. It is also in agreement with [135] who showed that *OPN-c* is expressed in breast cancer tissue but not in normal tissues, and that might be associated with tumor grade and disease progression. Also, another study demonstrated higher levels of OPN-c in breast cancer patients compared to its levels in the healthy control group [149]. OPN-c levels significantly increased with increases in the tumor grade ($p = 0.021$) as shown in figure (4.16) the result is in agreement with the results of [148] who reported that elevated *OPN-c* expression levels correlate with metastasis, advanced stage, and tumor recurrence. The results of the current study also confirm that in breast cancer, OPN-c is expressed in elevated mRNA levels compared to the corresponding levels expressed in the healthy tissues [212]. The selective expression of OPN-c in breast cancers, but not in healthy control samples may provide a simple reliable diagnostic and prognostic marker.

Table 4.8 Densitometric analysis comparing between both the OPN and OPN-c gene expression in six tissue samples

Patient No.	**OPN**	**OPN-c**
T1	2.3	2.5
T2	3.7	1.3
T4	3.2	3.7
T5	3.3	4
T7	2.8	4.1
T8	3.8	1.6

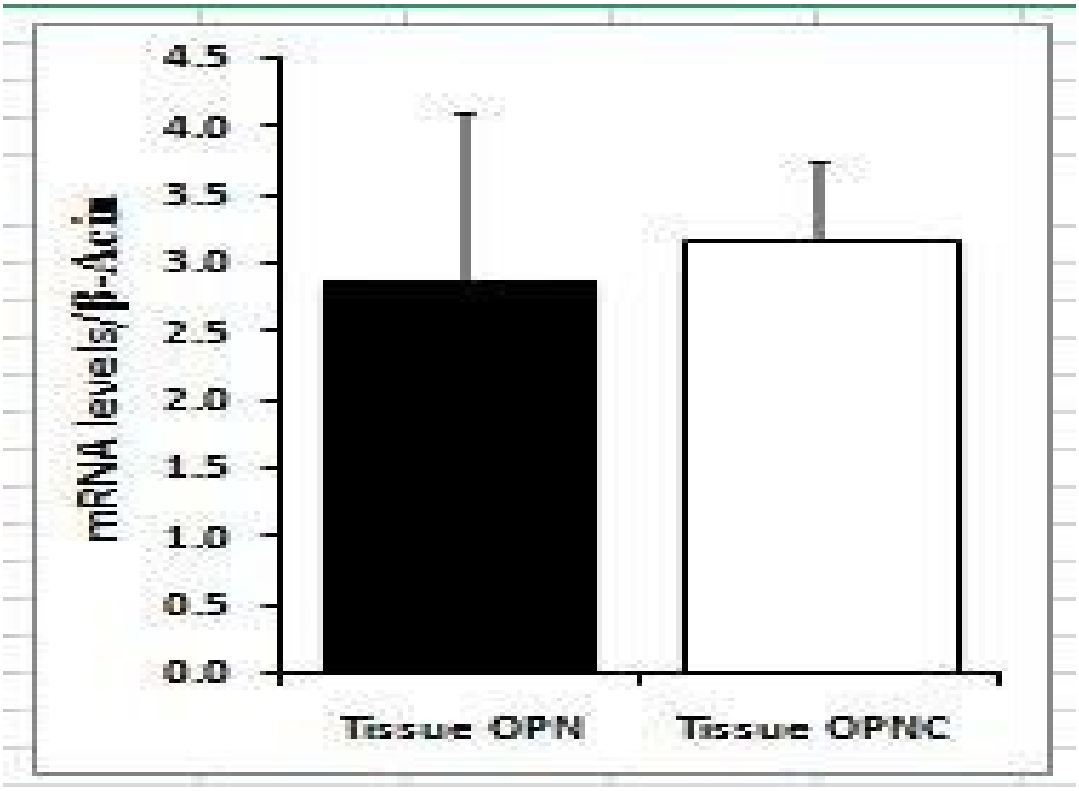

Figure 4.17 Analysis of *Osteopontin* and *Osteopontin-c* mRNA expression in tissue samples

Besides breast cancer, also *OPN-c* has been associated with the progression of other types of malignancies, including ovarian cancer, colon cancer, and hepatocellular carcinoma; this may suggest that OPN-c has the potential to be a marker of invasiveness not only for breast cancer but also for other types of malignancies. As shown in the table (4.8) and the corresponding analysis in figure (4.17) after comparing OPN expression levels with OPN-c expression levels in breast cancer tissues, it appears that there is no significant difference between OPN and OPN-c expression ($p > 0.05$). Both OPN and OPN-c are expressed at high levels in tumor tissues, but OPN-c is seen to be more reliable than OPN expression levels, especially when comparing the expression of the genes with tumor grade. Osteopontin-c is a better breast cancer marker than osteopontin

because the splice variant (OPN-c) was absent in the normal breast tissues examined.

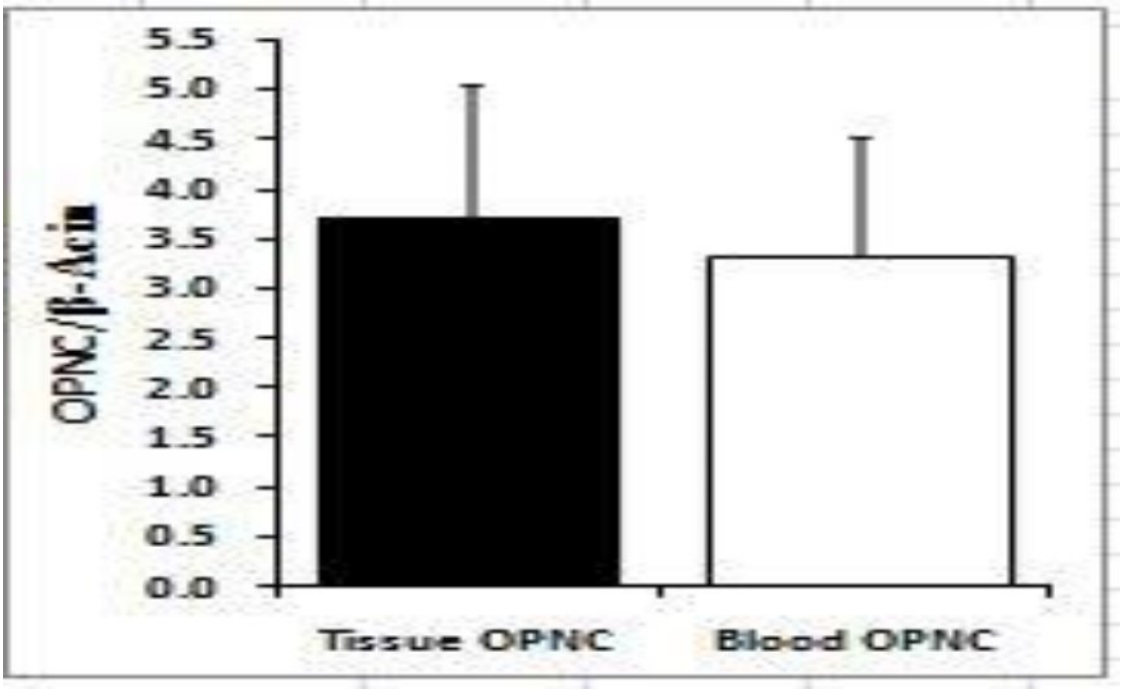

Figure 4.18 *Osteopontin-c* expressions in both tissue and blood from same patients.

In the present study, one of the goals was to investigate the similarities of gene expression in breast cancer tissue and their corresponding blood samples from the same patients for early detection of breast cancer. Breast cancer development occurs as a result of alteration in the expression of many genes. In both, the tissue and the blood, gene expression patterns can help in the definition of the biological processes that associated with profiles related to genetically controlled diseases [101]. Blood itself is critical for understanding the disease development and for the selection of better treatments. Thus, blood has the potential to be used diagnostically for direct sampling [137] as a pre-step for the following diagnosis.

To our knowledge, this is the first study that analyzes the *OPN-c* mRNA expression levels in both tissue and blood samples from the same patients. As shown in figure 4.28 there is no significant difference between Osteopontin-c mRNA expression levels in tissue and *OPN-c* mRNA in the blood ($p = 0.641$). The results suggest that OPN-c mRNA levels in the blood can be used instead of tissue samples, for screening and diagnosis of breast cancer; however, more studies with a large number of patients are required.

Chapter Five

Conclusions & Recommendations

Chapter Five

Conclusions and Recommendations

5.1. Conclusions

The following conclusions are drawn from the results

- Breast cancer disease is significantly associated with higher expression levels of *Osteopontin* and *Osteopontin-c* m RNA.
- *OPN* is detected in both normal tissues and tumor tissues with elevated levels of *OPN* mRNA in breast tumor tissues compared to the control.
- OPN measurement is shown to be a useful marker, and might be of greatest value when used in combination with conventional markers, including the(ER), (PR) and (Her)-2.
- *OPN-c* was detected in breast carcinomas while it was absent in normal breast tissues suggesting that *Osteopontin-c* might be the most clinically appropriate isoform of the Osteopontin gene for being an optimal marker for breast cancer disease than other isoforms because it is absent from normal breast tissues, which in turn reflects the diagnostic value of *OPN*.
- *OPN-c* significantly correlates with tumor grade; this makes *OPN-c* a candidate marker for the invasive potential of breast carcinomas which reflects its role in metastasis.
- *OPN-C* is detected in the blood of breast cancer patients with significantly elevated levels compared to healthy control. Thus, Osteopontin-c mRNA blood levels might be an additional option for monitoring cancer progression.

- **5.2. Recommendations**
- It is recommended that *OPN-c* might be used in the diagnosis of breast cancer in conjunction with other conventional markers.
- Further studies on *OPN* using larger sample size and more follow-up period are recommended.
- Targeting *OPN* might be a suitable therapeutic approach for the treatment of cancer; it will allow the development of novel therapeutic agents.
- Additional studies to better understand the signaling mechanism by which Osteopontin promotes tumorigenesis is recommended.
- Finally , in addition to the measurement of mRNA transcript levels by RT-PCR, a quantitative analysis of mRNA expression levels by real-time PCR is recommended as a more reliable technique for cancer detection and analysis of OPN protein expression.

Appendix

Appendix -1- Characteristics of the breast cancer patients blood samples

Sample code	Sample type	Age	Family History	Histology	Grade	β – actin	OPN	OPN-C
1b	Blood	45	NO	Invasive ductal carcinoma	I	Yes	Yes	NO
2b	Blood	48	NO	Invasive ductal carcinoma	II	Yes	Yes	NO
3b	Blood	41	NO	Invasive ductal carcinoma	III	Yes	Yes	NO
4b	Blood	44	Yes her sister	Invasive ductal carcinoma	II	Yes	Yes	NO
5b	Blood	42	NO	Invasive ductal carcinoma	III	Yes	Yes	NO
6b	Blood	47		Invasive ductal carcinoma	III	Yes	Yes	NO
7b	Blood	63	NO	Invasive ductal carcinoma	II	Yes	Yes	NO
8b	Blood	45	Yes her sister	Invasive ductal carcinoma	III	Yes	Yes	NO
9b	Blood	60	Yes her sister	Invasive ductal carcinoma	II	Yes	Yes	NO
10b	Blood			Invasive ductal carcinoma		Yes	Yes	NO
11b	Blood	37	Yes her aunt	Invasive ductal carcinoma	II	Yes	Yes	NO
12b	Blood	35		Invasive ductal carcinoma	II	Yes	Yes	NO
13b	Blood			Invasive ductal carcinoma		Yes	Yes	NO
14b	Blood			Invasive ductal carcinoma		Yes	Yes	NO
15b	Blood			Invasive ductal carcinoma		Yes	Yes	NO
16b	Blood	36	Yes her mother	Invasive ductal carcinoma	III	Yes	Yes	NO
17b	Blood	44	Yes her mother	Invasive ductal carcinoma	I	Yes	NO	Yes
18b	Blood	42	Yes	Invasive ductal carcinoma	II	Yes	NO	Yes
1a	Blood	45	NO	Invasive ductal carcinoma	I	Yes	Yes	NO

2a	Blood	48	NO	Invasive ductal carcinoma	II	Yes	Yes	NO
3a	Blood	41		Invasive ductal carcinoma	III	Yes	Yes	NO
4a	Blood	44		Invasive ductal carcinoma	II	Yes	Yes	NO
5a	Blood	42		Invasive ductal carcinoma	III	Yes	Yes	NO
7a	Blood	63		Invasive ductal carcinoma	II	Yes	Yes	NO
8a	Blood	45		Invasive ductal carcinoma	III	Yes	Yes	NO
9a	Blood	60		Invasive ductal carcinoma	II	Yes	Yes	NO
12a	Blood	35		Invasive ductal carcinoma	II	Yes	Yes	NO
13a	Blood			Invasive ductal carcinoma		Yes	Yes	NO
14a	Blood			Invasive ductal carcinoma		Yes	Yes	NO
15a	Blood	36	Yes her mother	Invasive ductal carcinoma		Yes	Yes	NO
16a	Blood		NO	Invasive ductal carcinoma	III	Yes	Yes	NO

Appendix -2- Characteristics of the breast cancer patients' tissue samples

Sample code	Sample type	Age	Family History	Histology	Grade	β – actin	OPN	OPN-C
T1	Tissue	43	NO	Invasive ductal carcinoma	II	Yes	Yes	Yes
T2	Tissue	35	NO	Invasive ductal carcinoma	II	Yes	Yes	Yes
T4	Tissue	39	NO	Granulomatous mastitis		Yes	Yes	Yes
T5	Tissue	49	NO	Invasive ductal carcinoma	III	Yes	Yes	Yes
T6	Tissue	37	NO	metastatic ductal carcinoma	III	Yes	no	Yes
T7	Tissue	39	NO	Invasive ductal carcinoma	III	Yes	Yes	Yes

T8	Tissue	49	NO	Invasive lobular carcinoma	I	Yes	Yes	Yes
T10	Tissue	43	NO	Invasive ductal carcinoma	I	Yes	no	Yes
T12	Tissue	44	NO	Invasive ductal carcinoma	II	Yes	no	Yes
T13	Tissue	40	NO	Invasive ductal carcinoma	I	Yes	no	Yes
T14	Tissue	60	NO	Invasive ductal carcinoma	III	Yes	no	Yes

Appendix -3- Statistical Analysis

No Correlation between Tissue OPN and Tissue OPNC

Correlation Coefficient (0.456)

P-value (0.364)

Descriptive Statistics

	Mean	Std. Deviation	N
Tissue OPN	2.8667	1.24043	6
Tissue OPNC	3.1833	.56362	6

Correlations

		Tissue OPN	Tissue OPNC
Tissue OPN	Pearson Correlation	1	-.456
	Sig. (2-tailed)		.364
	Sum of Squares and Cross-products	7.693	-1.593
	Covariance	1.539	-.319
	N	6	6
Tissue OPNC	Pearson Correlation	-.456	1
	Sig. (2-tailed)	.364	
	Sum of Squares and Cross-products	-1.593	1.588
	Covariance	-.319	.318
	N	6	6

No Correlation between Tissue OPNC and Blood OPNC

Correlation Coefficient (0.359) P-value (0.641)

Descriptive Statistics

	Mean	Std. Deviation	N
Tissue OPNC	3.7250	1.32004	4
Blood OPNC	3.3500	1.17898	4

Correlations

		Tissue OPNC	Blood OPNC
Tissue OPNC	Pearson Correlation	1	.359
	Sig. (2-tailed)		.641
	N	4	4
Blood OPNC	Pearson Correlation	.359	1
	Sig. (2-tailed)	.641	
	N	4	4

Significant Correlation between OPN-C levels and tumor Grade in tissue samples.

P-value (0.021), the mean difference is significant at the .05 level.

Descriptives

OPNC levels

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
Grade I	2	1.6000	.00000	.00000	1.6000	1.6000
Grade II	2	3.0000	.70711	.50000	-3.3531	9.3531
Grade III	2	4.0500	.07071	.05000	3.4147	4.6853
Total	6	2.8833	1.14441	.46720	1.6824	4.0843

Descriptives

OPNC levels

	Minimum	Maximum
StageI	1.60	1.60
StageII	2.50	3.50
StageIII	4.00	4.10
Total	1.60	4.10

ANOVA

OPNC levels

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.043	2	3.022	17.950	.021
Within Groups	.505	3	.168		
Total	6.548	5			

Multiple Comparisons

Dependent Variable: OPNC levels

	(I) Stages	(J) Stages	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	StageI	StageII	-1.40000	.41028	.083	-3.1145	.3145
		StageIII	-2.45000*	.41028	.019	-4.1645	-.7355
	StageII	StageI	1.40000	.41028	.083	-.3145	3.1145
		StageIII	-1.05000	.41028	.160	-2.7645	.6645
	StageIII	StageI	2.45000*	.41028	.019	.7355	4.1645
		StageII	1.05000	.41028	.160	-.6645	2.7645
Scheffe	StageI	StageII	-1.40000	.41028	.093	-3.1933	.3933
		StageIII	-2.45000*	.41028	.022	-4.2433	-.6567
	StageII	StageI	1.40000	.41028	.093	-.3933	3.1933
		StageIII	-1.05000	.41028	.176	-2.8433	.7433
	StageIII	StageI	2.45000*	.41028	.022	.6567	4.2433
		StageII	1.05000	.41028	.176	-.7433	2.8433
Bonferroni	StageI	StageII	-1.40000	.41028	.126	-3.3926	.5926
		StageIII	-2.45000*	.41028	.028	-4.4426	-.4574
	StageII	StageI	1.40000	.41028	.126	-.5926	3.3926
		StageIII	-1.05000	.41028	.250	-3.0426	.9426
	StageIII	StageI	2.45000*	.41028	.028	.4574	4.4426
		StageII	1.05000	.41028	.250	-.9426	3.0426
Sidak	StageI	StageII	-1.40000	.41028	.121	-3.3802	.5802
		StageIII	-2.45000*	.41028	.028	-4.4302	-.4698
	StageII	StageI	1.40000	.41028	.121	-.5802	3.3802
		StageIII	-1.05000	.41028	.230	-3.0302	.9302
	StageIII	StageI	2.45000*	.41028	.028	.4698	4.4302
		StageII	1.05000	.41028	.230	-.9302	3.0302

*. The mean difference is significant at the .05 level.

Homogeneous Subsets

OPNC levels

	Stages	N	Subset for alpha = .05	
			1	2
Tukey HSD[a]	StageI	2	1.6000	
	StageII	2	3.0000	3.0000
	StageIII	2		4.0500
	Sig.		.083	.160
Tukey B[a]	StageI	2	1.6000	
	StageII	2	3.0000	3.0000
	StageIII	2		4.0500
Scheffe[a]	StageI	2	1.6000	
	StageII	2	3.0000	3.0000
	StageIII	2		4.0500
	Sig.		.093	.176

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Significant correlation between OPN-C in blood of healthy and OPN-C in blood of breast cancer patients.

P-value (0.011) the mean difference is significant at the .05 level.

Descriptives

Blood levels of OPNC

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
Healthy	2	.6500	.35355	.25000	-2.5266	3.8266
Inflammation	2	2.0000	.42426	.30000	-1.8119	5.8119
Ca Breast	2	3.0500	.07071	.05000	2.4147	3.6853
Total	6	1.9000	1.10454	.45092	.7409	3.0591

ANOVA

Blood levels of OPNC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.790	2	2.895	28.016	.011
Within Groups	.310	3	.103		
Total	6.100	5			

Descriptives

Blood levels of OPNC

	Minimum	Maximum
Healthy	.40	.90
Inflammation	1.70	2.30
Ca Breast	3.00	3.10
Total	.40	3.10

Multiple Comparisons

Dependent Variable: Blood levels of OPNC

	(I) Status	(J) Status	Mean Difference (I-J)	Std. Error	Sig.
Tukey HSD	Healthy	Inflammation	-1.35000*	.32146	.049
		Ca Breast	-2.40000*	.32146	.010
	Inflammation	Healthy	1.35000*	.32146	.049
		Ca Breast	-1.05000	.32146	.092
	Ca Breast	Healthy	2.40000*	.32146	.010
		Inflammation	1.05000	.32146	.092
Scheffe	Healthy	Inflammation	-1.35000	.32146	.055
		Ca Breast	-2.40000*	.32146	.012
	Inflammation	Healthy	1.35000	.32146	.055
		Ca Breast	-1.05000	.32146	.103
	Ca Breast	Healthy	2.40000*	.32146	.012
		Inflammation	1.05000	.32146	.103
Bonferroni	Healthy	Inflammation	-1.35000	.32146	.074
		Ca Breast	-2.40000*	.32146	.015
	Inflammation	Healthy	1.35000	.32146	.074
		Ca Breast	-1.05000	.32146	.141
	Ca Breast	Healthy	2.40000*	.32146	.015
		Inflammation	1.05000	.32146	.141
Sidak	Healthy	Inflammation	-1.35000	.32146	.072
		Ca Breast	-2.40000*	.32146	.015
	Inflammation	Healthy	1.35000	.32146	.072
		Ca Breast	-1.05000	.32146	.134
	Ca Breast	Healthy	2.40000*	.32146	.015
		Inflammation	1.05000	.32146	.134

Homogeneous Subsets

Blood levels of OPNC

	Status	N	Subset for alpha = .05	
			1	2
Tukey HSD[a]	Healthy	2	.6500	
	Inflammation	2		2.0000
	Ca Breast	2		3.0500
	Sig.		1.000	.092
Tukey B[a]	Healthy	2	.6500	
	Inflammation	2		2.0000
	Ca Breast	2		3.0500
Scheffe[a]	Healthy	2	.6500	
	Inflammation	2	2.0000	2.0000
	Ca Breast	2		3.0500
	Sig.		.055	.103

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

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الخلاصه

يعتبرسرطان الثدي واحد من اهم المسببات الرئيسيه للامراضيه والوفيات عند النساء حول العالم. العامل الرئيسي المساهم للموت بسرطان الثدي هو وجود المرض الخبيث المنتشر,والذي هو السبب الرئيسي للموت ويمثل اكثر من 400000 حالة موت سنويا في كل انحاء العالم.لذلك,من المهم تحديد Biomarkersجديدة مع امكانية تطوير التشخيص المبكروالتنبؤ بتشخيص المريض,وتحسين مقاومة الدواء وخيارات العلاج. بينما Biomarkers لانواع معينه من السرطان لها اهمية, markers التي تحدد عدة سرطانات تكون اكثر قيمة في التشخيص والفحص ,حيث انه من غير العملي ان يكون Markerلكل سرطان محتمل.

ال Osteopontin هو بروتين سكري مفسفر وهو قادر على الارتباط بمستقبلات على سطح الخلية، ونظرا لخصائصه اللاصقه،ارتبط osteopontin مع تقدم أكثر من 30 نوع من السرطان.

تم تحديد ال marker في 60 عينة من منطقة كورستان /السليمانية / العراق ، منها 35 عينة دم من الإناث المصابات بسرطان الثدي، وعينات دم من 10 من النساء الأصحاء، و 15 عينة من نسيج النساء المشارياهم اثناء اجراء خزعة التشخيص بعد فحص الماموجرام المشبوه الأولي. وقد تم قياس مستويات التعبيرللرنا المرسال لل Osteopontin باستخدام عكس ناسخ البلمرة (RT-PCR) وقد لوحظ وجود فرق معنوي بين مستويات الرنا المرسال للاوستيوبونتين في دم مرضى سرطان الثدي مقارنة مع العينات السليمه ($P \leq 0.05$) ايضا اظهرت انخفاض ملحوظ في مستويات تعبيرال osteopontin بعد الدورة الأولى من العلاج الكيميائي مقارنة مع نفس العينات قبل العلاج الكيميائي ($P \leq 0.05$). وكانت مستويات osteopontin-ج الرنا المرسال في عينات الأنسجة ذات فرق معنوي عالي بين مرضى سرطان الثدي من الدرجة الاولى والدرجة الثالثه ($p = 0.021$) بينما لم يكن هناك فرق معنوي بين الدرجة الاولى والثانيه من مرضى سرطان الثدي ($p > 0.05$). ايضا لوحظ مستويات التعبير للرنا المرسال للosteopontin-ج كانت مرتفعه في دم مرضى سرطان الثدي مقارنة مع النساء الأصحاء. ($p = 0.01$). وكان الجزء المهم الرئيسي من هذه الدراسة المقارنة بين OPN-ج مستويات التعبيرللرنا المرسال في كل من النسيج والدم من

نفس مرضى سرطان الثدي والتي أظهرت عدم وجود ارتباط معنوي ($p = 0.641$) بين المجموعتين. وأشارت نتائج الدراسة إلى أن التعبير الجيني لل osteopontin ممكن ان يساعد بمثابة أداة تشخيصية وتنبؤية لرصد سرطان الثدي، وosteopontin-ج ك marker انتقائي لسرطان الثدي وأكثر موثوقية من osteopontin بسبب ان ال osteopontin –c كان غائبا في أنسجة الثدي الطبيعية.

التعبير الجيني للاوستيوبونتين في مرضى سرطان الثدي والاشخاص الاصحاء

رسالة

مقدمة الى مجلس كلية العلوم في

جامعة السليمانية كجزء من متطلبات نيل شهادة

ماجستير في علوم الحياة

(بايولوجي جزيئي)

من قبل

لينا ابراهيم كاظم

بكالوريوس التقنيات الحيوية (٢٠٠٥), جامعة بغداد

بأشراف

د. دلنيا اسعد

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ايار ٢٠١٦ شعبان ١٤٣٧

پوختە :

شێرپەنجەی مەمك دادەنرێت بە هۆیەکی سەرەکی نەخۆشکەوتن ولەناوچون لە ناو ژیاندا لە جیهاندا وە هۆکاری سەرەکی مردن بە شێرپەنجەی مەمك بە هۆی بلاوبونەوەی نەخۆشیە خراپەکانەوە (metastasis) , کە هۆکارێکی سەرەکی مردنە وەزیاتر لە (400 000) کەس لە ساڵێکدا بە هۆی ئەم نەخۆشیەوە دەمرن لە سەرتاسەری جیهاندا . بۆیە گرنگە (دەستنیشانکەری بایۆلۆجی) ی تازە دیاری بکرێت لەگەڵ توانای بەرەو پێشبردنی دیاریکردنی پێش وەخت و پێشبینی دیاریکردنی نەخۆشەکە و چاکردنی بەرەنگاربونەوەی بەدەرمان و هۆکارەکانی چارەسەرکردن. لەکاتێکدا (دەستنیشانکەری بایۆلۆجی) بۆ جۆرەکانی شێرپەنجە گرنگی خۆی هەیە بۆ دیاری کردنی جۆرەکانی شێرپەنجە وە زۆر بەبایەخەوە بۆ ناسین و پشکنین. لە کاتێکدا شتێکی کرداری نیە کە یەك marker بۆ هەر شێرپەنجەی پێشبینیکراو بەکاربێت.

osteopontin ئەو phosphorylated glycoprotein توانای هەیە بۆ یەکگرتنی لەگەڵ وەرگری سەر پەردەی خانەکان, وەلەبەر ئەوەی تایبەتمەندی بەیەکەوە لکاندنە *osteopontin* دێت لەگەڵ بەرەو پێشچونی زیاتر لە 30جۆر لە شێرپەنجە.

توانرا لە (60) نمونە marker دیاری بکرێت لە هەرێمی کوردستان _ سلێمانی, وە لە ناویان (35) خوێنی مەینە کەتوشبون بەشێرپەنجەی مەمك و (15) نمونە لە شانەی ئەو ژنانەی کە باسکران لە کاتی پشکنینیBiopsy لە دوای پشکنینی مامۆکرافی کە گومانیان لێ ئەکرا . وە توانرا ڕێژەی دەربڕینی *osteopontin* لە mRNA بپێورێت , بە بەکارهێنانی زیادکردنی reverse transcriptase .

وە لە ئەنجامی پشکنینەکاندا دەرکەوت کە دەربڕینی *osteopontin* لە هەموو نمونەکان وە بە ڕێژەی جیاوازدا. وە ڕێژەی دەربڕینی (mRNA) بۆ *osteopontin* بەلگەی سەرزمێری هەیە لە ئاستی 0.05 دا لە خوێنی نەخۆشەکانی شێرپەنجەی مەمك بەراورد بە نمونە تەندروستەکان , وە هەروەها دابەزینی بەرچاو لە پلەی دەربڕینی *osteopontin* دەرکەوت لە دوای خولی یەکەمی چارەسەری کیمیاوی, بەراورد بەهەمان نمونە پێش بەکارهێنانی چارەسەری کیمیاوی. وە پلەکانی (*osteopontin* - ج) ی mRNA لە نمونەی شانەکاندا بۆ جیاوازی مەعنەوی (0,021) p. valueلەنێوان نەخۆشەکانی شێرپەنجەی مەمك لەپلەی یەك و پلەی سێ.

هەروەها پلەکانی دەربڕینی (mRNA) بۆosteopontin– ج) بەرز بوو لە خوێنی نەخۆشەکانی شێرپەنجەی مەمك (0,011)p. value بەراورد بە ژنانی تەندروووست. و بەشی گرنگ وسەرەکی لەم خوێندنە بەراوردی نێوان (osteopontin– ج) ی پلەکانی دەربڕینی mRNA لە هەریەك لە شانەو خوێنی هەمان نەخۆشی شێرپەنجەی مەمك کە دەرکەوت هیچ پەیوەندیەکی مەعنەوی (0.641)p. value نیەلە نێوان ئەو دوو کۆمەڵەدا. وە ئەنجامی توێژینەوەکان دەریخست کە دەربڕینی جینی بۆ (osteopontin) ئەتوانێ یاریدەدەربێ بۆ پشکنین و پێشبینی لە دیاریکردنی شێرپەنجەی مەمك و osteopontin– ج هەڵبژێراوە بۆ شێرپەنجەی مەمك وە زیاتر باوەڕ پێکراوە لە osteopontin لەبەر ئەوەی کە (osteopontin–ج) دەرنەکەوتووە لە شانەکانی مەمکی ئاسایی .

دەربڕینی ئۆستیۆپۆنتین لە نەخۆشەکانی شێرپەنجەی مەمك و لەکەسانی لەش ساغدا

نامەیەك

پێشکەش کراوە بە ئەنجومەنی کۆلیجی زانست

لە زانکۆی سلێمانی وەك بەشێك لە پێداویستیەکانی بە دەستهێنانی بڕوانامەی

ماستەر لە زانستی بایۆلۆجی

(گەردی بایۆلۆجی)

لەلایەن

لینا ابراهیم کاظم

بەکالۆریۆس لە بایۆتە کنۆلۆجی (٢٠٠٥), زانکۆی بەغداد

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