Republic of Iraq
Ministry of Higher Education and Scientific Research
AL-Nahrain University
College of Science

MicroRNAs as Possible Biomarkers for Breast Cancer in Samples of Iraqi and British Women

A thesis
Submitted to the College of Science, University of Al-Nahrain as a partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biotechnology

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June
2014

Sha'aban
1435

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

الاحماض النووية الـ microRNAs كمؤشرات حيوية لسرطان الثدي في عينات لنساء عراقيات و بريطانيات

اطروحة مقدمة إلى كلية العلوم /جامعة النهرين
وهي جزء من متطلبات نيل درجة دكتوراه فلسفة في
علوم التقانة الاحيائية

من قبل

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بكالوريوس تقانة احيائية ٢٠٠٤
ماجستير تقانة احيائية ٢٠٠٧

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

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صدق الله العظيم

سورة الطلاق جزء من الاية ٢-٣

Dedication

I dedicate this effort to my wonderful family,
Thank you so much for all the love and support you have given me throughout my life and believing in me so I could believe in myself.
I had always wanted to study in the UK. You made that dream comes true. So thank you for that.
I appreciate your trust in my decisions and the guidance to get me their.
I dedicate this study also to everyone who will get the goodness of present work and apply it.

Zaynab
2014

Supervisor Certification

We, certify that this thesis entitled "**MicroRNAs as Possible Biomarkers for Breast Cancer in Samples of Iraqi and British Women**" prepared by "**Zaynab Saad AbdulGhany"** under our supervision at Department of Biotechnology/ College of Science/ Al-Nahrain University as a partial fulfillment of the requirements for the Degree of phD of Science in Biotechnology.

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Abstract

This study aimed to determine the expression of microRNA molecules as early prognosis biomarkers for the detection of breast cancer. To achieve this project, a total of fifteen serum samples were collected from Iraqi women with breast cancer and from six serum samples were collected from healthy women volunteers as a control group. In these samples, levels of estrogen and progesterone hormones and cancer antigen 15-3 (CA15-3) marker were determined to study the relationship between these parameters and breast cancer in Iraqi women. Results showed that there is a significant negative correlation ($r^2=^{-}0.4$) between the level of serum estrogen hormone and the age of breast cancer patients, while there is no significant correlation between the level of progesterone hormone and the age of the patients, as well as no significant correlation between the level of CA15-3 marker and patients age in comparison with healthy controls which means that estrogen hormone level was decreased with the age of patients.

On the other hand, the expression of several microRNAs in serum samples of breast cancer women and healthy controls were examined by using qPCR technique. These microRNAs included let-7a, miR-21, miR-222, miR-205, miR-218, miR-378, miR-15b, miR-26b, miR-27a, miR-429, miR-34a, miR-34b and miR-191(as endogenous control). Results showed that there is a significant increase ($P<0.05$) in the expression of let-7a and miR-21 in serum breast cancer patients in comparison with normal healthy controls with fold change (2.1 and 3.5) respectively, while there is significant downregulation ($P<0.05$) in the expression of miR-26b and miR-429 in serum of breast cancer patients compared with healthy volunteers with fold change (0.5) for both miRNAs that be regarded as novels miRNAs in detection of breast cancer. The rest of miRNAs did not show any significant variation on their expression in serum breast cancer patients and healthy women.

The expressions of miRNAs were also studied in thirteen plasma samples of British women with breast cancer and eight healthy normal volunteers. Results showed that there is a significant increase ($P<0.05$) in the expression of let-7a and miR-26b in plasma samples of breast cancer women compared with plasma of healthy women with fold change (1.5 and 2.0) respectively, while the expression of miR-27a and miR-222 were significantly down regulated ($P<0.05$) with fold change of (0.6) for both miRNAs.

On the other hand thirteen of matched British breast cancer tissues and non-cancerous tissues were used to determine the expression of miRNAs. The results revealed that miR-21 and miR-429 (the novel) were significantly increased ($P<0.05$) expression in breast cancer tissues compared to normal non-cancerous tissues with fold change (4 and 14) respectively, however significant downregulation ($P<0.05$) of miR-378 and miR-26b (the novel) expression has been detected in breast cancer tissues with fold change (0.2 and 0.6) respectively compared to its matched healthy normal non-cancerous breast tissues.

The last part of this study determined the expression of apoptotic genes in British breast cancer tissue samples and its matched non-cancerous normal breast tissues. These apoptotic genes included in this study are *P53*, *P21*, *Bax*, *Bcl2*, *BRCA2* and *TWIST* genes. Results indicated that the expression of *P53*, *P21* and *TWIST* were significantly downregulated ($P<0.05$) in breast cancer tissues compared to non-cancerous breast tissues with fold change 0.9, 1.0 and 1.0 respectively, while there is a significant upregulation ($P<0.05$) in the expression of *Bax* and *BRCA2* with fold change 3.5 and 20.0 respectively.

In conclusion, the results indicated that there is a possibility of detection the changes in the level of microRNAs expression as useful biomarkers for the detection and early diagnosis of breast cancer.

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List of Abbreviations

Abbreviation	Term
Δ	Delta
®	Registry Number
AD	Alzheimer disease
AFP	Alpha- fetoprotein
AKT	v-akt murine thymoma viral oncogene homolog 1
AML	acute myeloid leukemia
ATM	Ataxia telangiectasia mutated
BAX	B cell Associated X protein
BCL2	B-cell CLL/lymphoma 2
BCR-ABL	Break point cluster region- abelson
BMP	Bone morphogenetic protein
Bp	base pair
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
BTG2	B-cell translocation gene 2
CA15-3	Cancer antigen 15-3
c-AMP	Cyclic adenosine monophosphate
Cdc25a	Cell division cycle 25 homolog A
CDH	Cadherin
CDK	Cyclin Dependent Kinase
cDNA	Complementary Deoxyribonucleic acid
CEA	Carcinoembryonic antigen
c-kit	Trans membrane kinase
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
COPP	Chronic obstructive pulmonary disease
CSF	Cerebrospinal fluid
CT	Cycle Threshold
CV	Cardio vascular
DCIS	ductal carcinoma *in situ*
dNTPs	Deoxy nucleotide triphosphates

Abbreviation	Term
DSBs	Double strand breaks
EGF	Epidermal growth factor
EMT	epithelial to mesenchymal transition
ER	estrogen receptor
ER-α	estrogen receptor alpha
ERK	Extracellular signal regulated kinase
Fra-1	Fos- related antigen 1
FRA17B	Fragile site 17 B
GADD45A	Growth arrest and DNA-damage inducible 45 Gene
GIST	Gastrointestinal stromal tumor
Has	Homo Sapiens
HCC	Human breast cancer basal- like cell line
HCG-b	Human chorionic gonadotopin- beta
HER2	HER2 Human Epidermal growth factor Receptor 2
HMECs	human mammary epithelial cells
HNSCC	Head and neck squamous cell carcinoma
IHC	Immuno histo chemistry
ILC	Invasive lobular carcinoma
IMP	inosine 5- monophosphate
JNK1-3	Jun N terminal Kinase
Kip	Kinase inhibitor protein
K-RAS	Kirsten Rat Sarcoma
LCIS	Lobular carcinoma in situ
MAPK	mitogen-activated protein kinase
MCF7	Human Breast Adenocarcinoma cell line
MEK	Mitogen- activated protein kinase
miRISC	microRNA-induced silencing complex
miRNA	MicroRNA
miRNA*	MicroRNA complementary sequence
MMP2	Matrix metallo protein2
MMU	*Mus musculus*
mRNA	messenger Ribonucleic Acid
mTOR	Mammalian target of rapamycin

Abbreviation	Term
NSCLC	Non small cell lung cancer
ORF	Open reading frame.
OSCC	oral squamous cell carcinoma
OSTF1	Osmotic stress transcription factor 1
OVCAR3	Ovarian papillary adenocarcinoma cell
P21	Protein 21 kilo Dalton
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PDCD4	programmed cell death 4
PGA, C	Pepsinogen A,C
PGE2	Prostaglandin
PI3K	phosphoinositide3kinase
PR	progesterone receptor
pre-microRNA	precursor microRNA molecule
pri-microRNA	primary microRNA transcript
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog on chromosome 10
PTGs	Post transcriptional gene silencing
PTHRP	Parathyroid hormone related protein
q RT- PCR	Quantitative Real-time polymerase chain reaction
Raf	Rapidly accelerated fibrosarcoma
RCC	Renal cell carcinoma
RNA	Ribonucleic Acid
RNU48	Endogenous reference gene
RT	Reverse Transcription
SCC	Squamous cell carcinoma
SLC7A11	Solute carrier family 7
Sufu	Suppressor fused gene
TAD	Transcription-Activation Domain
TDLU	terminal ductal lobular unit
TEB	Terminal end buds
TGF-β	Transforming growth factor- beta
TNM	Primary tumor lymph node metastasis

Abbreviation	Term
TP53	Tumor Protein 53
TRAIL	Tumor-necrosis-factor related apoptosis-inducing ligand
UTR	Un translated region
VEGF	Vascular endothelial growth factor
WHO	World health organization
Wnt	wingless-type MMTV integration site family
ZBTB10	Zinc finger gene
ZEBl	zinc finger E- box binding homebox 1

1. Introduction

Breast cancer is one of the most common malignancies in women, and represents the second leading to death after heart failure worldwide (Jemal *et al.*, 2011). In fact, Iraq breast cancer ranks the first among the commonest malignancies among all the population and accounts for approximately one- third of the registered female cancer according to the latest Iraqi cancer registry (Iraq Cancer Board, 2010), which highlights the importance of early detection.

The diagnosis of breast cancer in women represents the major problems for early detection, accurate staging and monitoring of breast cancer (Ng *et al*., 2013). Thus, there is still need to develop a cost-effective and accurate screening method for this cancer and discover new biomarkers to improve diagnosis, prognosis and prediction (Bal and Hujol, 2006; Brase *et al*., 2010). As mentioned in American Cancer Society, (2012) the goal of screening for early breast cancer detection is to find the cancer before it starts to cause symptoms. Screening used to find a disease, such as cancer, in people who do not have any symptoms. Early detection means using an approach that lets breast cancer get diagnosed earlier than otherwise might have occurred.

Breast cancer can be diagnosed by careful physical examination, mammography, ultrasound (U/S), magnetic resonance imaging (MRI) and breast biopsy (Salsow *et al*., 2007). With recent technological advances, gene expression profiling is used also to detect early breast cancer and predict their prognostic outcomes (Harris *et al.*, 2007). After that, some biomarkers have been established in the routine evaluation of breast cancer; estrogen receptor ER (for predicting response to endocrine therapies) and Her2/neu (for predicting response to Transtuzumab)

(Thompson *et al*., 2008). Although these markers are assessed routinely but are far from perfect (Piccart-Gebhart *et al*., 2005).

MicroRNAs, which are novel class of naturally occurring, evolutionarily conserved, small RNA ranged in size 19-23 nucleotides, non coding RNA molecules and are important in gene regulation and expression either by inhibition of translation or degradation of mRNA (Lee and Ambros, 2001; Ambros, 2004; Nilsen, 2007).

Different studies and reviews mentioned the role of microRNAs in various biological processes such as their role in cell development, proliferation, apoptosis, differentiation and response to different stresses (Ioshikhes *et al*., 2007; Xia and Hu, 2010; Erbet and Sharp, 2012). Many studies (Calin and Croce, 2006a; Mitchell *et al*., 2008; Leidner *et al*., 2013) suggested that miRNAs are remarkably stable in blood stream against endogenous RNase activity and represent a novel promising blood based biomarker for cancer detection and diagnosis.

The increasing number of studies that prove the presence of miRNAs in circulating serum/plasma increases the chance of using these miRNAs as a good biomarker for cancer and other diseases (Cheng, 2012; Wang *et al*., 2012). For example, Koberle *et al*., (2013) and Ng *et al*. (2013) found that miRNA gene expression levels are aberrantly altered in breast cancer such as miR-21, miR-155 and miR-10b are over expressed; whereas miR-221 miR-125b and miR-145 are downregulated as mentioned by the studies of Iorio *et al*. (2005) and Xie *et al*. (2013) which highlight the importance of microRNA as a good biomarker in serum/ plasma of breast cancer patients.

It was expected in this study to detect the expression of some microRNAs in Iraqi breast cancer women to be used as an early diagnostic marker. Therefore, this study was designed to focus on microRNAs expression in breast cancer in different Iraqi and UK samples.

To achieve that goal, this study was aimed to:

1. Measuring the levels of estrogen and progesterone hormones in serum of Iraqi breast cancer and healthy women.
2. Detection of microRNAs molecules in the serum samples of Iraqi breast cancer and healthy women.
3. Detection of microRNAs molecules in British plasma samples of breast cancer and healthy women.
4. Detection of microRNAs molecules in British breast cancer tissue sections and its matched non-cancerous tissue sections.
5. Measuring the levels of circulating microRNAs in all samples by determining the fold expression of the detected microRNAs.
6. Determine the presence of some biomarkers associated with breast cancer by determining the apoptotic genes such as *p53*, *p21*, *Bcl2*, *Bax* and *TWIST*.

2. Literature Review

2.1. Cancer Biology

Cancer is a heterogeneous group of diseases characterized by uncontrolled growth of the cells. This proliferation if allowed to continue and spread could be fatal. About 90% of cancer related death is due to tumor spread as metastasis (Devita *et al*., 2011; Jain, 2013).

Cancer is a multi gene, multi step disease originating from single abnormal cell (DeSantis *et al*., 2011). The abnormalities in cancer cells usually resulted from mutation in protein coding genes that regulate cell division. Accumulation of mutations result cell grows abnormally and called tumors. Some cells in the tumors undergo further rounds of mutation leading to formation of malignant cells, which cause metastasis. Since malignant growth can occur in virtually all locations of the body, so that there are over 100 different types of cancers (Hejmadi, 2010).

Some properties of cancer cells are self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). The list has been further extended such as deregulating cellular energetic, avoiding immune response, tumor promoting inflammation and genome instability and mutation all together cause cancer to proliferate (Hanahan and Weinberg, 2011).

Not all tumors are cancerous; however tumor can be benign and malignant (American Cancer Society, 2012). Benign tumors are not cancerous, they can often be removed and in most cases they do not come back. Cells in benign tumors do not spread to other parts the body. While malignant tumors are cancerous, cells in these tumors can invade nearby tissues and spread to other parts of the body and this is called metastasis.

Some cancers do not form tumors for example; leukemia is a cancer of the bone marrow and blood (American Cancer Society, 2012).

The accumulation of DNA mutation and genome instability are the major factors for cancer to occur or develop. However, there are two types of factors to cause cancer, one of them is inherited traits which estimated that only 5-10% of cancer and the remaining 90-95% are either caused or contributed to by environmental factors or most cases is the combination of two (Anand *et al.*, 2008) as shown in figure (2-1).

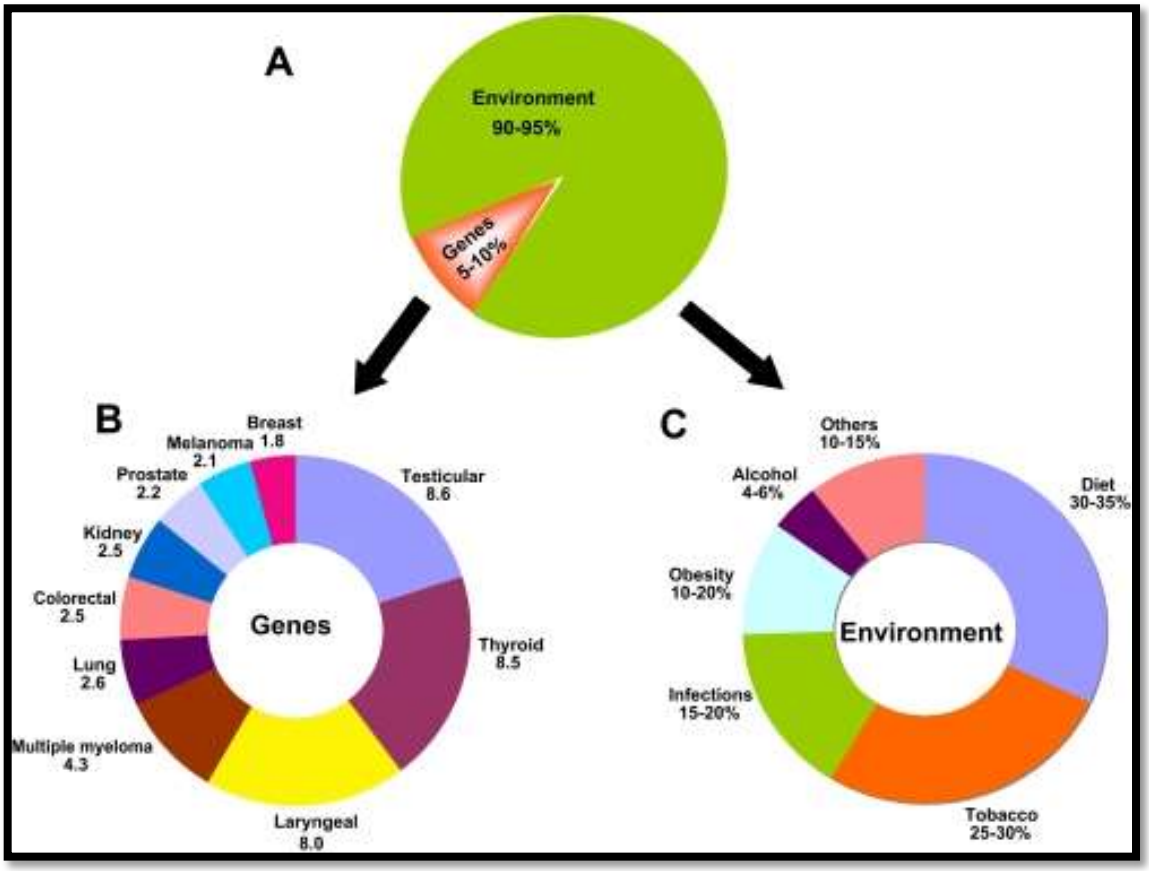


Figure (2-1): The impact of genes and environment on the development of cancer. Top figure shows the percentage contribution of genetic and environmental factors to cancer. Left: shows familial risk ratios - an age-adjusted risk ratio to first degree relatives of cases compared with the general population. Right: cancer deaths due to the specified environmental risk factor (Anand *et al.*, 2008)

Not only genome instability and genetic mutations develop tumors but also any environmental factors (for example exposure to x-ray) as presented in figure (2-2) that will affect on cell genome instability as well as influencing transcription, translation and posttranslational processes of the cell, leading to changing in genes- coding protein that regulate cell division leading to develop tumors or different phenotypic changes in the cell (Pamela and Douglas, 2010).

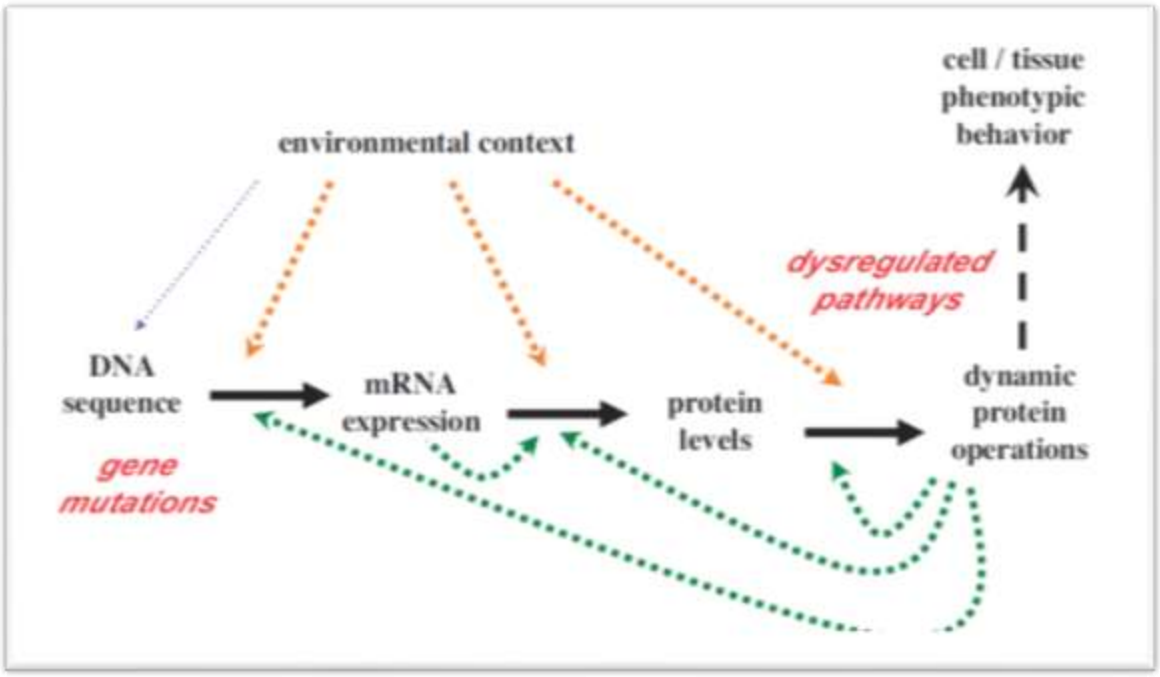


Figure (2-2): Central dogma. Transfer of genetic information from DNA, through mRNA to proteins. Any environmental and genetic mutations that will occur will affect on cellular pathways, which in turn affect on transcription and/or translation. This biological process is influenced by environmental factors as well (Pamela and Douglas, 2010)

2.1.1 Breast development

To understand the biological processes of human breast, it is important to understand normal breast tissue development. For *in vivo* studying of normal breast development, much of studies come from mice because of ethical use of human fetal tissue (Gusterson and Stein, 2012). It has been shown that mammary gland development in mice begins with the formation of fetal lens-shaped placodes that gradually form into a mammary ductal structure, also named an epithelial bud. After twelve days, the bud will consist of multiple epithelial cell layers, surrounded by mammary mesenchyme (Watson and Khaled, 2008).

In males, further mammary gland development is inhibited by androgens (male hormones), produced by the testes. In females, each bud starts to elongate to form a primary sprout. Outgrowth of the primary bud is induced by different developmental signals, including interactions between the parathyroid hormone-related protein (PTHrP) and bone morphogenetic protein (BMP) (Hens *et al.*, 2007). Subsequently, the primary sprout starts to penetrate the fad pad and a hollow lumen is formed. Primary ducts consisting of two epidermal layers develop secondary ducts which consisting of one epidermal layer. In total, humans approximately produce ten to twenty ducts. The ductal tree remains in the fat pad, consisting of a central and a basal layer surrounded by mesenchyme, which contributes to the formation of the nipple as described in figure (2-3), also in male (Tobon and Salazar, 1974; Chunha, 1994). At puberty, hormones are secreted by the ovaries or pituitary gland to initiate ductal elongation. At ductal elongation, terminal end buds (TEBs) appear at the tips of the ducts that proliferate and continue to invade the fat pad (Watson and Khaled, 2008).

The myoepithelial cells are in contact with the basement membrane and surround luminal epithelial cells (Beha *et al*., 2012). These cells do not only play a role in lactation, contracting milk out of the ducts, but are also thought to regulate luminal epithelial cell polarity, branching and differentiation (Sternlicht *et al.*, 1997; Bissell *et al.*, 2005).

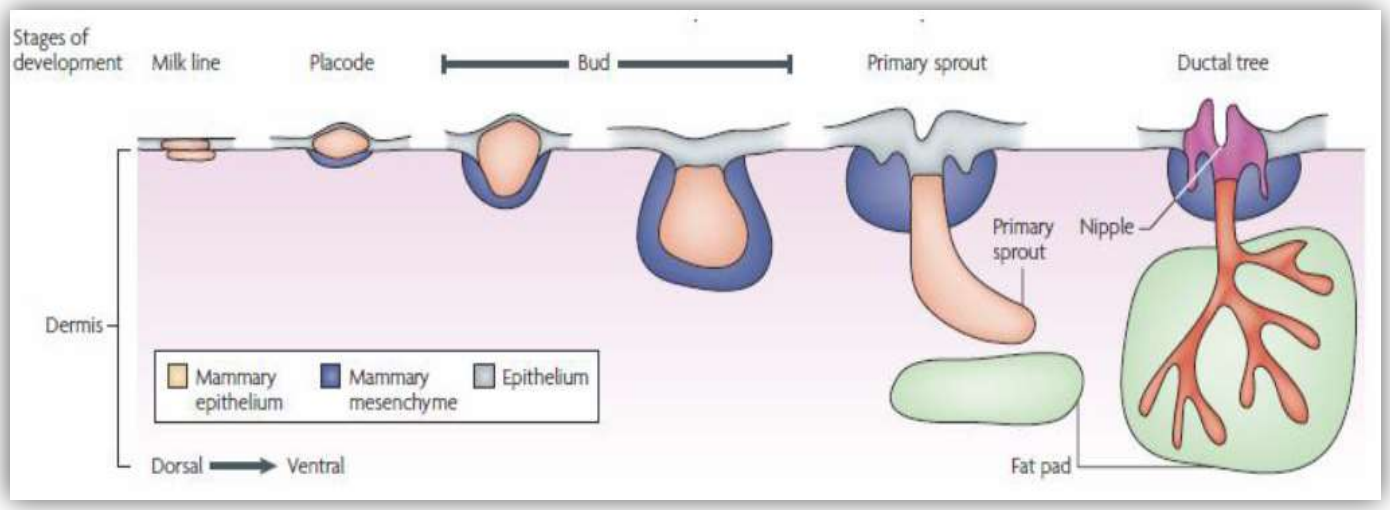


Figure (2-3): Stages of prenatal breast development. During fetal stages, placodes consisting of mammary epithelium give rise to an epithelial bud, surrounded by mesenchyme. Mesenchymal-epithelial interactions induce the primary sprout to invade the fat pad and form a ductal tree. The nipple is connected to the ducts, which is formed by the mesenchyme. The ductal tree remains in the fat pad with low activity, until puberty. This figure was taken according to (Robinson, 2007)

Estradiol and progesterone ovarian hormones are key regulators of mammary gland development (Felica *et al*., 2013). The majority of proliferating cells are found in the luminal epithelia of the breast. Likewise, estrogen and progesterone receptors are exclusively found in the luminal epithelium. After puberty, hormones regulate the formation of alveolar buds. However, full development of the alveoli and maturation of the mammary gland takes place during pregnancy (Medina, 1996; Anderson *et al.*, 2007).

2.1.2 Breast Cancer

Breast cancer now is the commonest female malignancy in almost all of Asia, Europe and North America (Ferlay *et al*., 2013) and suggested to be the first cause of death from cancers in women in Europe. Each year more than 1.3 million women were diagnosed with breast cancer worldwide and approximately 465,000 die from the disease (Gracia, 2007; DeSantis *et al*., 2011), despite the fact that breast cancer is highly curable if diagnosed and treated appropriately at an early stage (Irland National Cancer Registry, 2008).

The increasing incidence of breast cancer results in a critical need to identify and develop more sophisticated and patient-specific means of diagnosing and treating the disease, in order to minimize its associated morbidity and mortality.

2.1.2.1 Breast cancer pathology

Breast cancer is a heterogeneous disease caused by interaction of both inherited and environmental risk factors that leads to a progressive accumulation of genetic and epigenetic changes in breast cancer cells (Antoniou and Easton, 2006; Pamela and Douglas, 2010).

Each breast has 15 to 20 sections called lobes, which have many smaller sections called lobules. The lobes and lobules are connected by thin tubes, called ducts as shown in figure (2-4) (Devita *et al*., 2011).

The most common type of breast cancer is ductal cancer (Allegra *et al*., 2010). It is found in the cells of the ducts. Cancer that starts in lobes or lobules is called lobular cancer (Yoder *et al*., 2007). Cancers also are classified as non invasive (*in situ*) and invasive (infiltrating). The term *in situ* refers to cancer that has not spread out the area where it initially developed. While invasive breast cancer has a tendency to spread

(metastasize) to other tissues of the breast and/or other regions of the body (Devita *et al*., 2011).

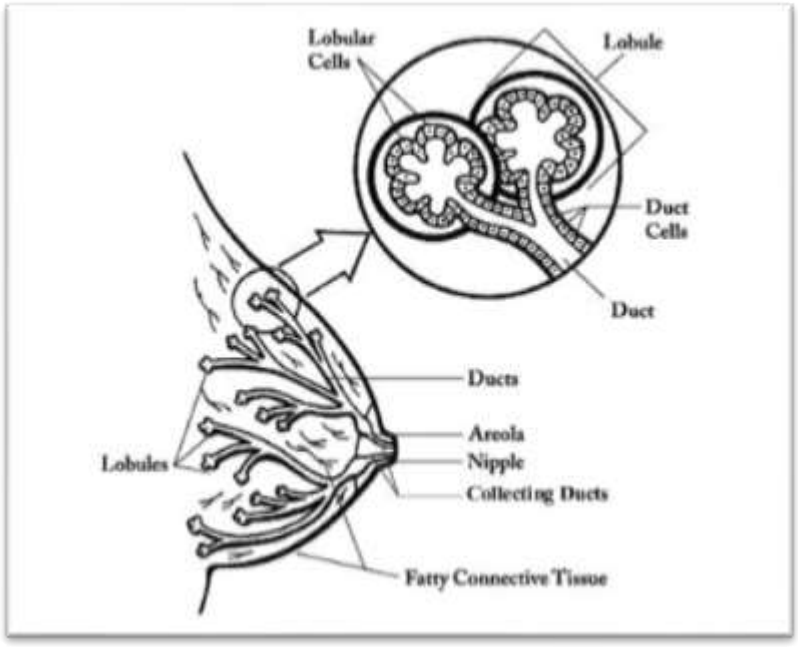


Figure (2-4): The structure of the female breast (Devita *et al*., 2011)

More than 95% of breast cancers arise from the breast epithelial elements. However the term "breast carcinoma" encompasses a diverse group of lesions which differ in microscopic appearance and biological behavior (Dabbs, 2012). Breast carcinomas can be divided into three major groups classified according to the location of the tumor (Peter and Fattaneh, 2003):

1. ***In situ* non- invasive carcinomas**: the tumor cells remain confined to the ducts or lobules and show no evidence of microscopic invasion into the surrounding breast stroma (Devita *et al*., 2011).There are two types of *in situ* carcinoma; ductal and lobular, named according to the predominant cell type from which the tumor arises. Because non-invasive type represents the common in women with breast cancer, the present section will briefly describe non invasive carcinoma, which includes:

♦ **Ductal Carcinoma *In Situ:* DCIS**

It represents 70% of non-invasive breast carcinoma. More than 60% of patients with DICS are recognized by mammography as microcalcification, while the others presented as palpable mass or nipple discharge (Devita *et al*., 2011; Lee *et al*., 2012). DCIS characterized as proliferating malignant ductal cells within breast without invasion through the basement membrane (Devita *et al*., 2011).

♦ **Lobular Carcinoma *In situ*: LCIS**

It represents multi centric in 70% of the cases and 30-40% bilateral and has no distinguishing features. LCIS defined as obliteration and distension of the lobule by a relatively uniform, round, small to median sized cells with round and normo chromatic nuclei (Devita *et al*., 2011; Butler and Rosa, 2013).

♦ **Ductal Carcinoma *In Situ* with Paget's disease:**

Here the same morphology of DCIS with the presence of Paget's cells in the epidermis of nipple (Muttarak *et al*., 2011). These cells are large hyper chromatic cells, having pleomorphic nuclei surrounded by a clear halo which represent the intracellular accumulation of muco polysaccharides (Al-Naib, 1970).

2. Invasive carcinomas: the tumor cells invade the breast stroma and have the potential to spread beyond the ducts or lobules. The invasive breast carcinomas consist of several histological subtypes; the commonest being infiltrating ductal adenocarcinoma (75-80%), followed in frequency by invasive lobular (10-15%), Mixed ductal-lobular (<5%), Inflammatory (2-3%), Colloid (2-3%), Tubular (<2%), Medullary (<2%), and Papillary (1%) (Li *et al.*, 2005; Rakha *et al*., 2010; Devita *et al*., 2011).

3. Metaplastic breast cancer account for less than 5% of overall cases. Invasive breast cancer metastasizes via local invasion to chest wall or skin, lymphatic infiltration to axillary nodes most commonly, or

haematogenous spread to distant sites including bone, liver, lung and brain (Devita *et al*., 2011).

2.1.2.2 Breast cancer staging

The current methods of breast cancer patients staging into prognostic groups, which influences therapeutic decisions, are based on clinicopathological parameters such as size and grade of the tumour, presence or absence of lymph node metastases, and distant metastases so that is called TNM staging, as indicated in table (2-1). The gold standard method in routine use is the TNM staging system adapted from Singletary and Connolly, (2006).

Table (2-1): Clinical staging group of breast cancer, were T: primary tumor present, N: any lymph node detected and M: if the tumor metastasis out from the breast to other organs.

Stage	T.N.M. staging	Prognosis (5 years overall survival)
Stage 0	Tis, N0,M0	93%
Stage I	T1, N0, M0	88%
Stage IIa	T0 or T1, N1, M0 or T2, N0,M0	
Stage IIb	T2,N1,M0 or T3,N0,M0	74-81%
Stage IIIa	T0-T1, N2,M0 or T3,N1 or N2, MO	
Stage IIIb	T4, N0-N2, M0	41-67%
Stage IIIc	T, N3, M0	
Stage IV	Any T, any N, M1	15%

Tumor, Nodal and Metastasis (TNM) classification from breast cancer according to American Joint Committee on Cancer Staging as presented in table (2-2) as mentioned by Devita *et al*.,(2006).

Table (2-2): Tumor, Nodal and Metastasis (TNM) staging of breast cancer.

Grade	Description
T grading	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma *in situ* eg. DCIS, LCIS, and Paget's disease
T1	Tumour _ 2 cm
T2	Tumour > 2 cm, _ 5 cm
T3	Tumour > 5 cm
T4	Tumour of any size with direct extension to chest wall or skin
N Grading	
NX	Regional lymph nodes cannot be assessed (eg. removed previously)
N0	No regional lymph node metastases
N1	Metastases in 1-3 movable ipsilateral axillary lymph node(s)
N2	Metastases in 4-9 ipsilateral axillary nodes, fixed or matted axillary nodes, or metastases in ipsilateral internal mammary nodes
N3	Metastasis in 10 or more axillary lymph nodes, or in infraclavicular lymph nodes, or in clinically apparent ipsilateral internal mammary nodes in the presence of one or more positive axillary lymph nodes; or in more than three axillary nodes with clinically negative microscopic metastasis in internal mammary lymph nodes; or in ipsilateral supraclavicular lymph nodes
M grading	
MX	Presence of distant spread (metastasis) cannot be assessed
M0	No distant metastases
M1	Distant metastases present (sites commonly include bone, lung, brain, liver).

2.1.2.3 Breast Cancer Risk Factors

Nowadays every woman is at risk for developing breast cancer because of presence of strong risk factors that affect large percentage of population (Kelsey and Gammon, 1990; American Cancer Society, 2012).

Most known risk factors for breast cancer are age, previous breast cancer, benign breast disease, hereditary factors (family history of breast cancer), early age at menarche, late age at menopause, late age at first full-term pregnancy, low physical activity and high-dose exposure to ionizing radiation early in life (Marc *et al.*, 2007; American Cancer Society, 2012).

The other suspected risk factors for breast cancer include never having been pregnant, having only one pregnancy rather than many, not breast feeding after pregnancy, use of postmenopausal estrogen replacement therapy or postmenopausal hormone (estrogen/progestin) replacement therapy, use of oral contraceptives, certain specific dietary practices (high intake of fat and low intakes of fiber, fruits, and vegetables, low intake of phytoestrogens), alcohol consumption, tobacco smoking, and abortion (Dumitrescu and Cotarla, 2005; Phipps *et al*., 2011).

Although men can and do develop breast cancer, the disease is 100 times more likely to occur in a woman than in a man (Wu *et al.*, 2002; American Cancer Society, 2012), because they have much more breast tissue than men do, also estrogen (hormone that secreted from ovary) promotes the development of breast in women.

2.1.2.3.1 Age:

The risk of breast cancer is higher in middle-aged and elderly women than in young women (Edwards *et al.*, 2002). This risk increases as a woman ages rising after the age of 40 (American Cancer Society, 2012).

2.1.2.3.2 Family history:

A woman who has previous history of breast cancer has three to four folds at increased risk of developing a new cancer in the other breast. Women who have had benign breast problems are also at increased risk but to a lesser extent (Helmrich *et al.,* 1983; Minami *et al.*, 1999). The risk of breast cancer is higher among women who have a close blood relative (mother, sister or aunts) who have had the disease (Bevier *et al*., 2012). The increase in risk is especially high if the relative developed breast cancer before the age of 50 or in both breasts (Claus *et al.,* 2003). The effect of family history on breast cancer risk is believed to be due primarily to genetic factors.

2.1.2.3.3 Mutation:

It has been shown that germ line mutations in the BRCA1 and BRCA2 genes account for a large proportion of cases of hereditary breast cancer (Ford and Easton, 1995; Domchek *et al*., 2010). As much as 5–10 % of all breast cancer cases are attributable to specific inherited single-gene mutations, and many other cases have some genetic component.

2.1.2.3.4 Estrogen production:

Women who reach menarche at a relatively early age (12 or younger) and those who reach menopause at a relatively late age (55 or older) are slightly more likely than other women to develop breast cancer (Helmrich *et al.*, 1983). These relationships are believed to be mediated through estrogen production (Henderson *et al.*, 1988; Misser *et al*., 2004).

During the reproductive years, a woman's body produces high levels of estrogen. Women who start to menstruate at an early age and/or reach menopause at a late age are exposed to high levels of estrogen for

more years than are women who have a late menarche or early menopause (Kendall *et al*., 2007; Chlebowski *et al*., 2010).

2.1.2.3.5 Pregnancy:

Age at first pregnancy is another point of reproductive history that is associated with breast cancer risk (Nechuta *et al*., 2010). Women who have their first full-term pregnancy at a relatively early age have a lower risk of breast cancer than those who never have children or those who have their first child relatively late in life (Helmrich *et al.*, 1983). The biologic basis for this relationship is not entirely clear.

Parity (having children) and the age of the woman at the birth of her first offspring are other endogenous hormonal factors that influence breast cancer. Woolcott and coworker (2012) presented that women who have never had children (nulliparous) are at greater risk for the development of breast cancer than women who have had children (parous). There is also consistent evidence that first pregnancy completed before age 30-35 lowers risk of breast cancer, and that first full-term pregnancy after age 30-35 raises risk. More limited evidence suggests that women who have many pregnancies may be less likely to develop breast cancer than those who have only one pregnancy (Ayyappan *et al*., 2010).

In some studies, premature termination of pregnancy appears to increase breast cancer risk (Newcomb *et al.*, 1996). In incomplete pregnancy, the breast is exposed only to the high estrogen levels of early pregnancy and thus may be responsible for the increased risk seen in these women. However, some other studies found no association between abortions and increased risk of breast cancer (Erlandsson *et al.*, 2003; ACOG Committee Opinion , 2009). While other studies shown that women who breast-feed their babies may be less likely to develop breast

cancer than those who have children but do not breast-feed (Katsouyanni *et al.*, 1996).

2.1.2.3.6 Radiation:

Women who were exposed to high doses of radiation, especially during adolescence, have an increased risk of breast cancer (Linct *et al*., 2012). This association has been observed both among atomic bomb survivors and among women who received high-dose radiation for medical purposes (Preston *et al.*, 2002).

The long-term (more than five years) use of postmenopausal estrogen therapy (ERT) or combined estrogen/progestin hormone replacement therapy (HRT) may be associated with an increase in breast cancer risk (Porch *et al.*, 2002; Phipps *et al*., 2011). A dose-response relationship was found in women who had been treated with X-rays and who had a family history of breast cancer (Adams *et al*., 2011).

2.1.2.3.7 Diet:

A possible relationship between breast cancer and diet has been suggested due to the variation of breast cancer in societies with different national diets (the high rates in Western industrialized nations and the low rates in Asia, Latin America, and Africa) (McTiernan *et al*., 2010). A comparison of vegetarian versus meat-eating women produced inconclusive results. And the effects of fiber, fruits, and vegetables now appear to be small, at best. Diets high in fruits and vegetables and low in fat and calories are healthful for many reasons, and they may indirectly reduce the risk of breast cancer by helping to prevent obesity (Pike *et al*., 1999).

2.1.2.3.8 Obesity:

Obesity has been consistently associated with an increased risk of breast cancer among postmenopausal women (Protani *et al*., 2010; Anderson and Neuhouser, 2012). This relationship may be mediated again by estrogen production (Cleary and Grossman, 2009). Fat cells produce some estrogen and obese postmenopausal women, therefore, tend to have higher blood estrogen levels than lean women (Hirose *et al.*, 2001; Brown and Allen, 2002).

2.1.2.3.9 Smoking:

There is some evidence that cigarette smoking may be associated with a small increase in breast cancer risk (Xue *et al*., 2011). However, epidemiological studies have variably shown positive, inverse, or null association (Palmer and Rosenberg, 1993). Among women who have already been diagnosed with breast cancer, smoking may be associated with an increased risk that the cancer will progress more rapidly.

2.1.2.4 Tumor Markers

A tumor marker is defined as a substance present/overexpressed in or produced by a tumor (tumor-derived), or the host (tumor-associated), that can be used for differentiating neoplastic from normal tissue. Tumor markers are found in cells, tissues, and body fluids such as cerebrospinal fluid, serum, plasma, and milk (Etheridge *et al.*, 2011).

The ideal marker would be useful in diagnosis, staging and prognosis of cancer, provide an estimation of tumor aggressiveness, and serve for monitoring effects of therapy, detecting recurrence, localization of tumors, and screening in general populations (Heneghan *et al.*, 2010a).

Most tumor markers uses nowadays do not fit the ideal profile because the relative lack of sensitivity and specificity of the available

tests. It should be noted that any protein or chemical has the potential to be a tumor marker. As tumor cells grow and multiply, some of their substances increase in tumor tissues and/or leak into the bloodstream or other fluids and can examine that to detect any new biomarker and useful for early diagnosis of disease (Heneghan *et al.*, 2010b).

Depending upon the tumor marker, it can be measured in blood, urine, stool or tissue. Some widely used tumor markers include: AFP, Her2/Neu, beta-HCG, CA 19-9, CA 27.29 (CA 15-3), CA 125, CEA, and PSA (Harris *et al.*, 2007) and some were shown in table (2-3). Some tumor markers are associated with many types of cancer; others, with as few as one. Some tumor markers are always elevated in specific cancers; most are less predictable. However, no tumor marker is specific for cancer and most are found in low levels in healthy persons, or can be associated with non-neoplastic diseases as well as cancer (Heneghan *et al.*, 2010a).

The next section will pass quickly about major biomarker uses by laboratories for early diagnosis of cancers:

2.1.2.4.1 Carcinoembryonic Antigen (CEA) and Cancer Antigen (CA15-3):

They are cell surface glycoprotein and they represent markers for colorectal, gastrointestinal, lung, and breast carcinomas (Bates and Longo, 1987). CEA and CA 15-3 is most useful in monitoring therapy (as declining levels correlate with tumor aggressiveness) and has utility in detecting recurrence of colorectal cancer. High CEA levels in breast cancer do not correlate with grade of tumor but are useful for monitoring therapy and detecting recurrence (Uehara *et al.*, 2008).

2.1.2.4.2 Human epidermal growth factor receptor 2 (HER2/neu):

It is an oncogene-encoded growth factor receptor (homologue of epidermal growth factor (EGF) receptor), also known as c-erbB-2. It is overexpressed in breast cancers as a result of HER 2 proto-oncogene amplification (Crown *et al*., 2013). It is measured in the tissue from a biopsy either by immunological assays of the protein or PCR. The presence of HER-2/neu is generally associated with a more aggressive growth and poorer prognosis for breast and ovarian cancer (Tiwari *et al.*, 1992; Lindgren *et al*., 2008). It can also help to determine treatment options predicting an enhanced survival benefit from the Her 2-targeted therapy reviewed by (Horton, 2002).

2.1.2.4.3 Estrogen receptor (ER) and progesterone receptor (PR):

In both pre- and postmenopausal women, levels of steroid receptors ER and PR can predict which women are likely to benefit from hormone treatment. Measurements of ER and PR are recommended to use in the diagnosis, prognosis, and treatment planning for women with breast cancer (Thompson *et al.*, 2008). ER gives an indication of responsiveness to therapy. Tissue from a biopsy is used to measure the estrogen receptor. Most breast cancers in post menopausal women are ER-positive, meaning that they require estrogen to grow. These ER positive breast cancers are less aggressive than ER negative breast cancers, which are found generally in premenopausal women (Saltzman *et al*., 2012).

Table (2-3): Some commonly biomarkers used in standard and clinical practice according to Alex, (2006)

Marker	Clinical use	Anti cancer agent	Specific disease	Methodology
APF	Predictive and prognostic marker	Non-specific	Hepatoma /germ cell tumor	ELISA
BCR-ABL	Predictive and prognostic marker	Non-specific	CML	PCR
c-Kit	Predictive and prognostic marker	Non-specific	GIST, CML	IHC
CA125	Predictive and prognostic marker	Non-specific	Ovarian cancer	ELISA
CEA	Predictive and prognostic marker	Non-specific	Colon cancer liver metastasis	ELISA
ER	Predictive and prognostic marker	Non-specific	Breast cancer	IHC
Her2/Neu	Predictive and prognostic marker	Non-specific	Breast cancer, Ovarian cancer	IHC, FISH
B-HCG	Predictive and prognostic marker	Non-specific	Germ cell tumor	ELISA
p-ERK	PD marker	MEK, Raf kinase inhibitor	Multiple tumors	IHC, Western blotting
PSA	Predictive and prognostic marker	Non-specific	Prostate cancer	ELISA
P70S6K	PD marker	mTOR inhibitors	No	IHC, Western blotting
Prelamin A/HDJ-2	PD marker	FTIs	Multiple tumors	IHC, Western blotting

2.1.2.4.4 P53:

Represent a tumor suppressor gene that is mutated or changed in more than 50 percent of tumors. Studying p53 as a tumor marker helped researchers understand how tumors form, but measuring p53 levels in cancer patients has not been shown to predict differences in survival or quality of life (Osyka and Ishioka, 2007; Kumar *et al*., 2012). Level of p53 was indicated as responsible for tamoxifen resistance in breast cancer suggesting that it can interfere in treatment response (Guillot *et al*., 1996).

Researchers continue working on specific molecular pathways involved in oncogenesis, tumor response, tumor progression, etc. to discover new molecular markers that can have a potential to be routinely used in medical practices of breast cancer.

2.1.2.5 Current diagnosis approaches of breast cancer

There are three major techniques that are commonly used to evaluate breast masses excluding surgical procedures. It has been estimated that from 8-38% of breast carcinoma can't detected by palpation alone (Rosai, 2004).

1. Physical breast examination:

This is inexpensive, non invasive method of detection that can be taught to patients. It has been show that approximately 60-90% of all breast cancers are usually found either by patients themselves or by examining physician or nurse (Nelson *et al*., 2009).

2. Mammography:

Today represent the available method to detect breast cancer in its earliest, most treatable stage in an average of 1.7 years before the woman can feel the lump (Taplin *et al*., 2008). The success of routine screening for breast cancer is that it involves increasingly more patients with small primary tumors formerly though to have an overall excellent prognosis.

3. Pathological examination

The removed biopsy from the breast must be examined microscopically by a pathologist. The pathologist will look for any abnormalities in the cells such as, shape, size, structure and the borders of the cells to decide whether these cells are malignant or not (WHO, 2006; Lester, 2010).

Well, most of the used methods are employed to detect visible tumors, the important things is to search more to find any markers that detect un-visible one with high specificity and sensitivity.

2.1.2.6 *BRCA* genes associated with breast cancer development

Genetic, hormonal and environmental factors each have role in breast cancer. Inherited mutations in *p53*, *BRCA1* and *BRCA2* are known to confer a predisposition to breast cancer (Holstege *et al*., 2009). Most molecular studies of breast cancer have focused on just one or two high information content platforms, most frequently mRNA expression profiling or DNA copy number analysis and more recently massively sequencing has been used (Balmana *et al*., 2011). The recent development of additional high information content assays focused on abnormalities in DNA methylation, miRNA expression and protein expression, provide further opportunities to more completely characterize the molecular of breast cancer (Cortes *et al*., 2013).

BRCA1 and BRCA2 are highly associated with breast cancer predisposition genes identified by genome- wide linkage analysis and position cloning (Konishi *et al*., 2011). Mutations in BRCA1 and BRCA2 explain 20% of the familial clustering of breast cancer. The prevalence of BRCA1 or BRCA2 germ line mutations varies considerably among ethnic groups and geographical areas (Fackenthal and Olopade, 2007).

BRCA1 was first cloned in 1994, followed by BRCA2 in 1995 (Narod and Foulkes, 2004). Both of these genes play role in preventing cancer from developing, specifically in the breast and ovaries. A mutation in one of these genes leads to an increased risk of breast and ovarian cancers (Teng *et al*., 2006). Both located on chromosome 13 and both belong to a class of genes known as tumor suppressors (Narod and Foulkes, 2004). BRCA1 protein product is involved in DNA damage repair. While BRCA2 involved in the repair of chromosomal damage with an important role in the error-free repair of DNA double strand breaks (Yoshida and Miki, 2004).

Women who have an abnormal BRCA1 or BRCA2 gene have up to an 85% risk of developing breast cancer. by age over 40; increased risk of developing ovarian cancer is about 55% for women with BRCA1 mutations and about 25% for women with BRCA2 mutations (Tapia *et al*., 2008)

Pal and collegues (2010) suggested that numerous mechanisms alter the process of cell cycle, division and DNA repair and given that it may be affected by BRCA1 and BRCA2 mutations may limit rather than promote reproduction.

2.1.2.7 MicroRNA

2.1.2.7.1 Discovery, Nomenclature and Biogenesis:

To start talking about microRNA it is possible first to view its starting point which leads to identify these small molecules. Early in 1990, two scientists Lee and Ambros and their colleagues worked on *Caenorhabditis elegans* when they found the role of lin-4 and lin-14 genes. They found that these genes play a role in developmental control of *C. elegans* (Lee *et al.*, 1993). After several years later Reinhart and his colleagues discovered the second microRNA in *C. elegans* called let7, which play a vital role in developmental timing in these warms (Reinhart *et al.*, 2000).

MicroRNAs are small 19-23 nucleotides, non protein coding RNA; binds to complementary sequence of target mRNA causing either repression of translation, target degradation or gene silencing (Lee *et al.*, 1993). The sequence homology of micrRNAs between species gives indication of importance of these molecules in several biological functions (Ambros, 2004).

Later on, spot light on microRNA were occurred and more studies were carried out, one of these are discovery of new microRNA species by Calin and his colleagues and found the correlation between microRNA expression and human disease specially cancer in B cell leukemia (Calin *et al.*, 2002), figure (2-5) presents the time line for microRNA discovery.

Furthermore, Rosenfeld and his group showed that microRNA expression is very important in detecting tissue-specific cancer, which highlight the role of microRNA as a biomarker for cancer diagnosis (Rosenfeld *et al.*, 2008).

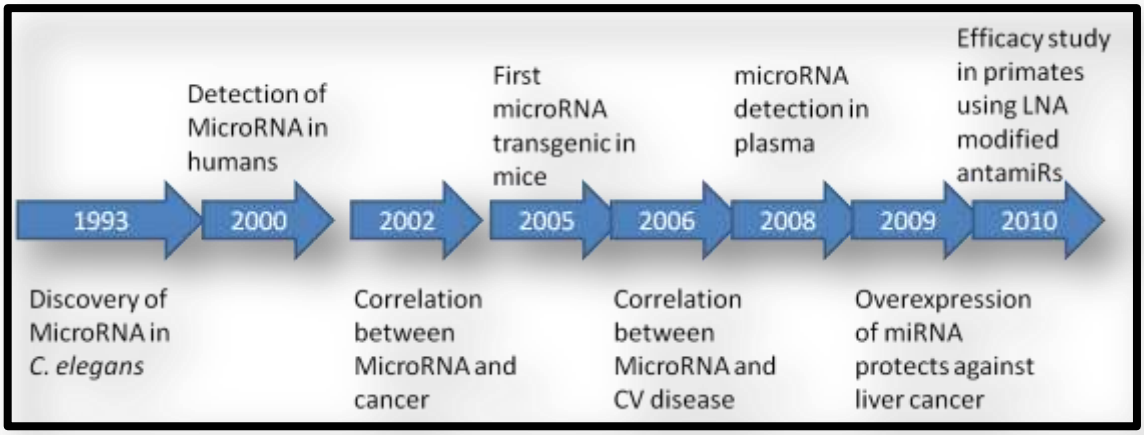


Figure (2-5): Time line of microRNAs discovery. This was adopted from Rooji, (2011)

One of the first studies that measure microRNAs level in serum of patients with prostate cancer were carried out, indicates the possibility of presence of these molecules circulate in blood (Mitchell *et al.*, 2008), which open the way to diagnose and predict the presence of cancer and use these molecules as a biomarker.

Griffiths-Jones and colleagues (2006) mentioned that the names of microRNAs are signed as numerical order and are provided by miR-Base registry, which is an organization hosted and maintained in the Faculty of Life Science at Manchester University with funding from Biotechnology and Biological Science Research Council.

As shown in table (2-4), miRNA genes are preceded with three to four letter prefix to identify the species of origin (Mikaelian *et al.*, 2013), for example, the prefix "mmu" *Mus musculus* miRNAs, while "has" for *Homo sapiens* miRNAs. The product of gene transcription, which is the primary miRNAs, is labeled "pri miRNA", while "pre miRNA" is for precursor miRNAs. Letter suffix identify mature miRNAs with high homology. Number suffix identify mature miRNAs that originate from different regions of the genome but have the same sequence. There are few

exceptions to this nomenclature, the let7 family was named "let" for "lethal" because knock-outs *C. elegans* are not viable (Mikaelian *et al.*, 2013).

Table (2-4): Nomenclature of microRNAs adopted from (Mikaelian *et al.*, 2013)

Symbol	Definition
Prefix: has and mmu	Indicates species
miR-XXX	All microRNA are identified by 3-4 numbers
Pri-miR	Primary miRNA: gene transcript with hairpin loop structure, capped with a specially modified nucleotide at the 5′ end, and polyadenylated, may contain 1-6 pre-miR
Pre- miR	Precursor miRNA: hairpin structure obtained from the pri-miRNA and is characterized by two nucleotide overhang at its 3′ end
miR-XXX	Mature miRNA that is the guide strand "-3p" anti sense
miR-XXX*	Passenger strand "5-p" sense
miR-XXXa and miR-XXXb	Indicates high homology
miR-XXX-1and miR-XXX2	Identical mature miRNAs originating from different regions of the genome
Seed sequence	Nucleotides 2-7 of miRNA complementary to target mRNA

To understand how microRNAs affect on protein synthesis and degrade mRNA, must first show how these small molecules produced inside the cells as shown in figure (2-6), Bernardo and coworker (2012) present starting from the nucleus: microRNAs are transcribed the RNA polymerase II to gives a primary transcript (pri-miRNAs). The cleavage of this pri-miRNA is processed by endogenous enzyme called Dorsha, resulting pre-miRNAs with 72 nucleotides.

This pre-miRNA will export to cytoplasm by the assessing of an enzyme called exportin5. In the cytosol: RNA III endonuclease, Dicer, this

enzyme will recognize pre-miRNA and cleave its loop, leaving 5′-phosphate and 2-3 nucleotides in 3′-end. The miRNA duplex now separated to give two stands one called guide strand which is antisense and this strand will integrated into the RISC, RNA inducing silencing complex where complementary mRNA are degraded, and the other miRNA strand is called the passenger strand and designated as miR*, which has a sense orientation, this will degraded, figure (2-6) simplify this description (Kim and Nam, 2006).

Like mRNA, some microRNA differentially expressed among tissues or developmental stages. Because of their restricted expression profiles these microRNAs hold promise as diagnostic markers or therapeutic targets for tissue or biological stage specific diseases (Lanford *et al.*, 2010).

MicroRNA binds with target mRNA, and then inhibit mRNA from the translation process. From this sequence specific binding, an average microRNA may have hundreds of target mRNAs, this binding tell us that the regulation of a mRNA target by a specific miRNA not only depend on the concentration of mRNA but also on the concentration and availability of many other mRNAs which are less complementary (Brennecke *et al*., 2005).

2.1.2.7.2 MicroRNA circulating freely in blood

Generally microRNA proved to be a stable molecule with half lives of hours to days in the liver and heart (Krol *et al*., 2010). Unlike RNA which its half life in plasma is short because of presence of ribonucleases enzymes that degrade RNAs, which expected by studies these enzymes degrade 99% of circulating RNA within 15 seconds (Tsui *et al.*, 2002), miRNA remain stable even in harsh conditions because of the envelope or packaging particle called exosome.

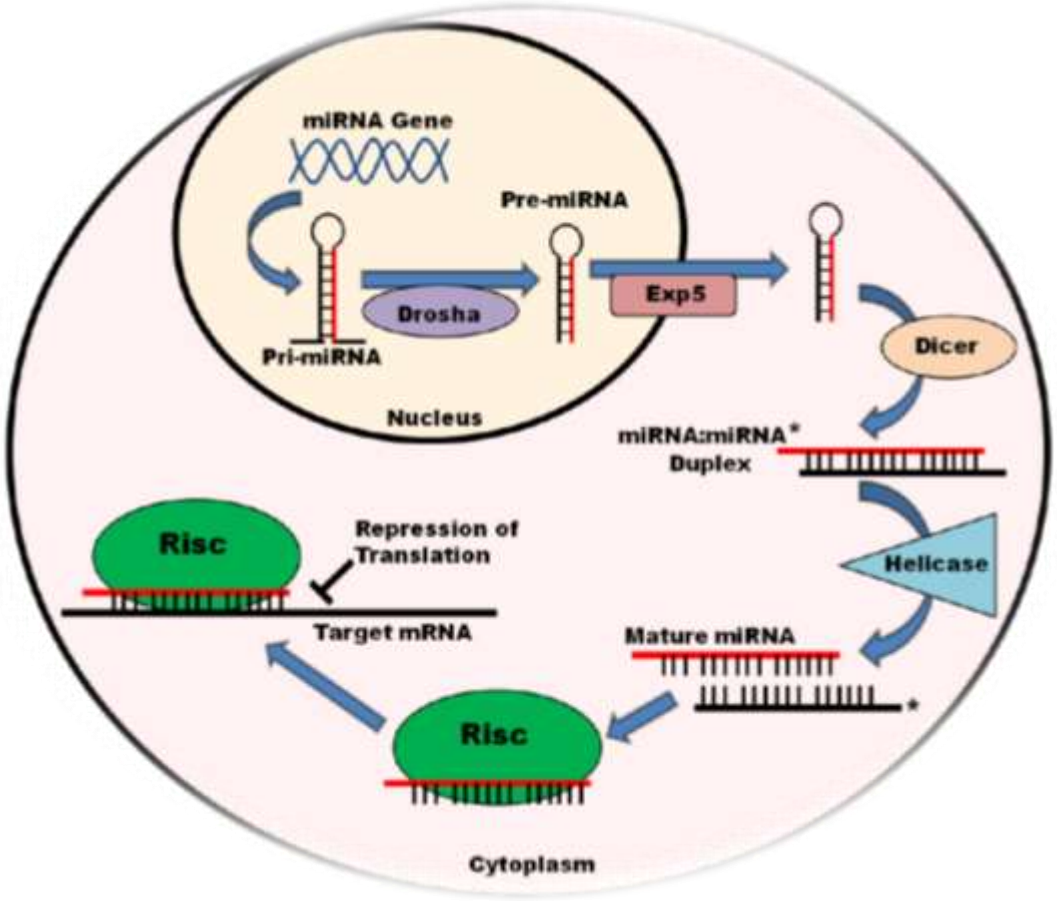


Figure (2-6): Simplified microRNA biogenesis according to Kim and Nam, (2006)

El- Hefnawy and colleagues (2004) showed that plasma microRNA is protected from degradation by inclusion in lipid or lipoprotein complexes. One of the first reports showing the existence of miRNA in exosome was by Valadi *et al.*, (2007) who reported that exosome released from human as presented in figure (2-7) and murine mast cell lines contains RNAs and miRNAs.

In addition to protecting against degradation, these exosomes function to upload and deliver miRNAs which capable of affecting in gene expression in specific recipient cells.

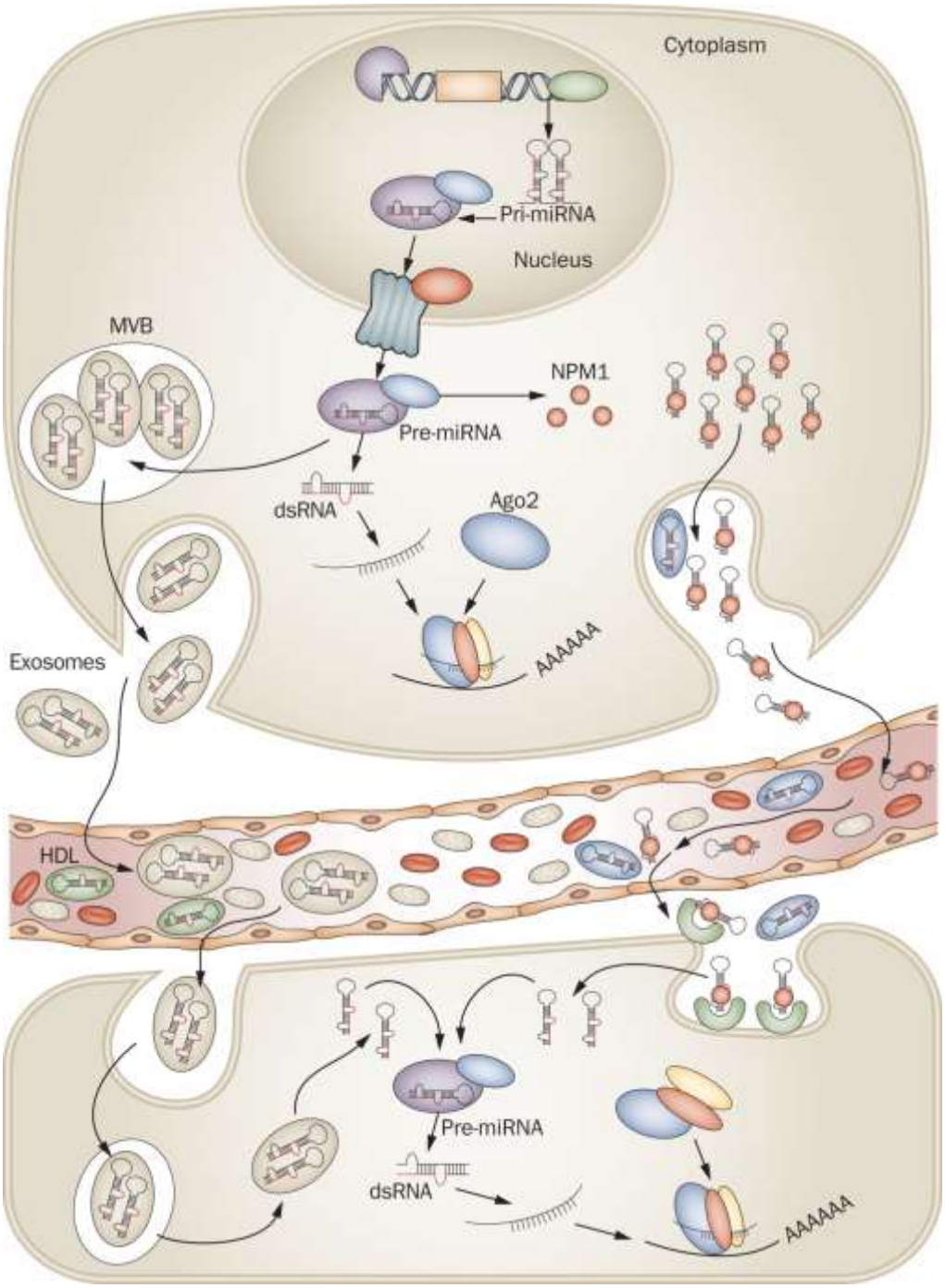


Figure (2-7): MiRNAs produced from donor cell and packaged in exosome and transferred through blood stream to another recipient cell. MVB: multivesculare bodies, NPM1: nucleophomin1and HDL: high density lipoprotein. (Valadi *et al.*, 2007)

Two types of cell derived lipid vesicles as presented in figure (2-7): microvesicles and exosomes. Microvesicles are relatively large (100 nm to 1μm) and released from cell through blebbing, while exosomes are smaller in size about (30-100 nm) released when endosomally derived multivesicular bodies fuse with plasma membrane. MicroRNA has been identified in both types (Valadi *et al.*, 2007).

Exosomes in saliva, plasma and breast milk, all contains RNA which supports the idea that RNA can be transferred from cell to another and also represents type of cell- cell communication (Lasser *et al.*, 2011). Several studies proved that exosomes are secreted by different cells including cancer cell, dendritic cells, macrophages, T and B cells and mast cells (Thery *et al.*, 1999; O'Neill and Quah, 2008).

There are many studies that show highly abundant of microRNAs in blood of patient with different types of cancers. For example, Lawrie *et al.*, (2008) the first who discover tumor specific deregulation of circulating miRNA -21 in serum of diffuse large B-cell lymphoma patients. Ji and his colleague (2009a) determined plasma concentration of miR-208 increased significantly after isoproterenol induced myocardial injury in rat.

Hu and colleagues (2010) performed a screening study to detect serum miR to predict the diagnosis of non small cell lung (NSCLC) using Solex sequencing. The occurrence of specific miRNA profiles associated with tumor grade and decreases in the circulating concentrations of specific miRNA following treatment of prostate cancer were determined in a study of Brase *et al.*, (2011), show the value of circulating microRNA as a biomarkers. Thus plasma miRNAs may be used as biomarkers to assess response to treatment in orthotropic and transgenic models of malignancies.

The ideal properties of biomarker should be disease specific, able to differentiate between pathologies, rapid and significant release during

pathology development, long life in sample, rapid, simple, accurate and inexpensive for detection, un affected by environmental conditions, present in accessible body fluid (Etheridge *et al.*, 2011) .

2.1.2.8 MicroRNA and Cancer:

Early evidence of miRNA role in cancer based on studies carried on *C. elegans* and Drosophila, and showed how these miRNA controls cell proliferation and apoptosis in these (Lee *et al.*, 1993; Brennecke, 2003).

The first studies about miRNA expression in human cancer were performed by Calin *et al.*, (2005). The study was used to examine a recurring deletion at chromosome 13q14 to search for tumor suppressor genes involved in chronic lymphatic leukemia (CLL). The result show that this region at the chromosome were encodes two miRNAs; miR-15a and miR-16-1, and the scientists then confirmed that these two miRNAs have role in CLL pathogenesis.

Calin and his coworker (2004) found that genes for miRNA were located at fragile sites in the genes which are frequently amplified or deleted in human cancer. The data of the study provide a catalog of miRNA genes that may have roles in cancer and argue that the full complement of miRNA in genome may be involved in cancer.

MiRNA involved in different biological process and pathological cases especially in development of several cancers. Any altered expressions in levels of miRNA have been correlated in different studies with cancer type, tumor stage and response to treatment. These studies confirm that miRNA represent a new class of biomarker as a diagnostic and prognostic and cancer therapy (Bartels and Tsongalis, 2009).

MiRNA-196 and miRNA-10a were shown to be located in homebox clusters, which is known to be involved in carcinogenesis and it is associated with the malignant of cancer cell (Chen and Sukmar, 2003).

Each miRNA is thought to target multiple genes this will affect cellular process and may induce carcinogenesis.

Up and down regulation of miRNA expression were observed in tumor compared to normal tissues, this indicate that microRNA play as either oncomiRs or tumor suppressors (Lu *et al*., 2005).

Generally genes encoding miRNAs were located on chromosomal regions that are amplified or over expressed in cancer and function as oncogenes, while those are deleted or less expressed in cancer act as tumor suppressor miRNAs (Miska, 2005). Uncontrolled proliferations, loss of apoptotic activity, promote angiogenesis and / or invasion and tumor formation are representing how miRNA act as oncogenes.

Over expression of miR-21 were observed in all cancers, which support the evidence of the role of this miR in malignancies and its oncomiR activity. And there are numbers of tumor suppressor genes were targeted by this oncomiR like PTEN (Lou *et al.*, 2010).

The role of miR-143 was studied in human bladder and colon cancer, and shown to be significantly lower in tumor compared to normal tissues. This miRNA was shown to inhibit cell proliferation, which support the idea of its role as tumor suppressor (Lin *et al*., 2009).

Not only the miRNA have role in cancer and also in different diseases for examples; diabetes mellitus, obesity, heart failure, infections, inflammatory and auto immune diseases including hepatitis and rheumatoid arthritis (Erson and Petty, 2008). These studies show the role of miRNA in the pathogenesis of these diseases (Heneghan *et al.*, 2010c).

2.1.2.9 MicroRNA and Breast Cancer:

Because the present techniques in private hospitals are not sensitive, non specific like the use of mammography instead how efficient and how quick get result but it not accurate and there are a lot of factors affect the result one of these are the thickness of breast patient also its using of radiation even in small wavelength it may also affect the individual workers (WHO, 2006). Some tests like estrogen, progesterone and CA15-3 are inadequate used for more than one cancer and their levels were correlated with patient life style. FNA its very difficult to tell the patient we have to take a biopsy from her, it's very hard and painful and there a definite increase the possibility to get contamination after take the biopsy (Devita *et al*., 2011). So the urgent need for specific, accurate, high sensitive, easy gated, differentiated between cancer types. By this way scientist work hardly to find these small molecules that circulates freely in blood.

Focusing on expression of microRNA and its role in breast cancer are the result of development of new detection methods to detect these small molecules and new techniques to easy the studies with high degree of accuracy.

The first studies that determine the global miRNA expression profiles in breast cancer were come from the study of Iorio *et al*., (2005), who used 76 breast tumors and 34 normal. The result of the study showed that there are many miRNA which down regulated in these breast tumors like; miR-10b, miR-125b and miR-145 while others were shown to be up regulated like; miR-21and miR-155 as shown in figure (2-8). Another finding from this study, it can differentiate between normal and cancer breast tissues.

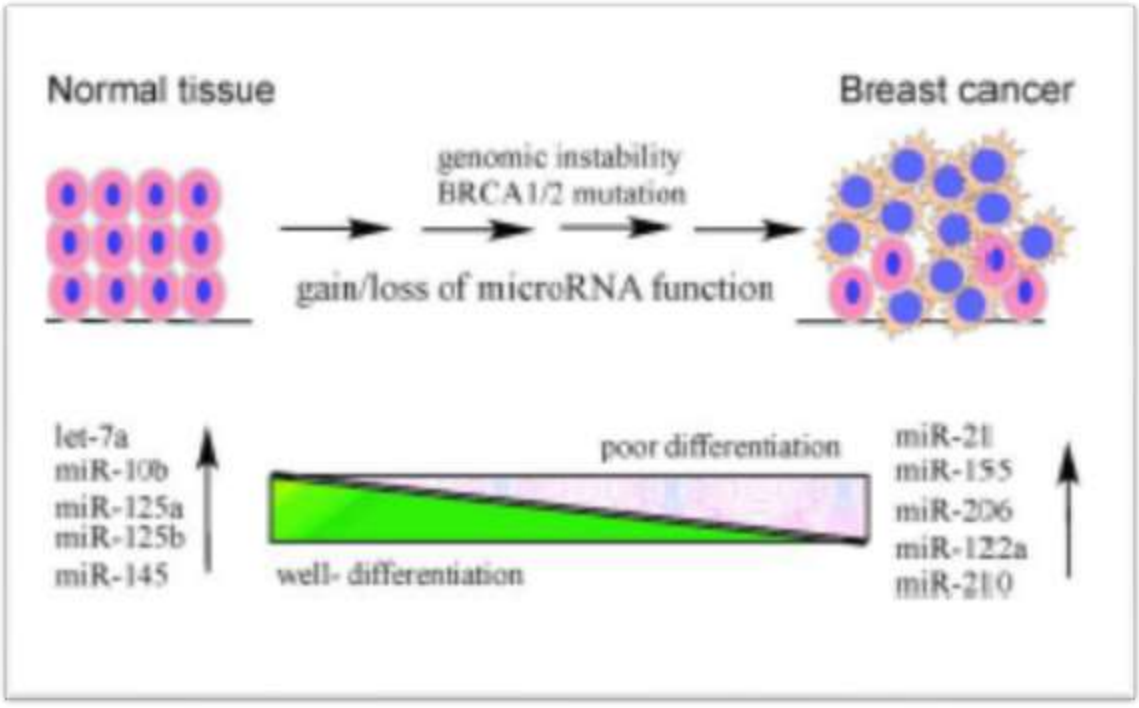


Figure (2-8): Expression of microRNAs in normal and cancer breast tissues (Iorio *et al*., 2005)

Other studies were carried out and one of them carried by BlenKiron and Goldstein, (2007), who showed that there is significant correlation between miRNA expression and tumor differentiation status, disease stage and patient outcome. Their study concluded that miRNA expression is very important as a new diagnostic biomarker in the future of breast cancer.

MiRNA expression was also shown to be altered when cell resistance or sensitive to drug used as chemotherapy in breast cancer patients. Climent and Dimitrow (2007) the first who indicates possible relation between miRNA deregulation and the development of drug resistance in breast cancer.

Interestingly, more publications then carried out and viewed the correlation between miRNA levels and ER, PR or ErbB2 status. Lowery and coworker (2009) demonstrated that miRNA 342, miR-290, miR-217, miR-190, miR-135b and miR-218 were strongly associated with ER

status; while miR-520g, miR-377, miR-527-518a and miR-520f-520c were associated with PR status. Lastly the study showed that miR-520d, miR-181c, miR-302c, miR-376b and miR-30e have roles in ErbB2 status.

One of the most studied microRNA is miR-21 which was found play role as oncogene in different cancers and in breast cancer specially (Selcuklu *et al.*, 2009). Knockdown of miR-21 in MCF7 breast cancer cell lines were shown to reduce the growth and reduce tumor growth in mouse xenograft (Si *et al*., 2007). While a study carried by Qian , (2009) show miR-21 over expression were correlated with disease aggressiveness and tumor grade.

By comparing the differentiation levels of miRNA expression in tumor cells, it could be expected that their levels were affected by methylation and demethylation of miRNA promoter regions during development of cancer. However, miRNA may mutate in breast cancer, which may affect the detection of its expression. So, the study of Wang and Wei (2007) declared that genomic instability, epigenetic changes and mutation of miRNA cause miRNA deregulated in breast cancer.

The role of miRNA in cancer invasion and metastasis were studied by Huang *et al.*, (2008) who found the role of two miRNAs, miR-373 and miR-520c, in promoting invasion and metastasis of MCF7 cell lines.

On the other hand, miRNA levels and expression were measured in serum of breast cancer patients and used to differentiate between healthy and cancer individual (Zhao *et al.*, 2010); and concluded from this study that serum microRNA could be used as a biomarker in early stage breast cancer.

In a study of Schrauder and his group (2012), they used microRNA microarray technology to analyze miRNA expression in whole blood of early stage breast cancer patients. The result indicated that miRNA have a diagnostic and prognostic potential in those patients compared to normal

controls. Depending on their results whole blood miRNA profiling could be used to measure the disease specific derived miRNA and circulating cell free miRNAs.

The stability of microRNAs, tissue specific expression profiles and the easy to be quantified, suggest that these molecules represent ideal biomarker for cancer. Elevated level expression of miR-195 with let7a and miR-155 in blood of breast cancer patients increase the specificity and sensitivity of the test (Heneghan *et al.*, 2010d). The result at the end concluded the potential utility of microRNA as unique and non-invasiveness breast cancer markers.

The research to identify more efficient miRNA biomarkers in blood is a nonstop process. The latest publications suggest that miR-92a in the circulation of serum breast cancer patient were elevated and correlates with tumor size and lymph node metastases (Si *et al.*, 2013). The study was gives some clues for improving diagnosis, prognosis and therapeutic strategy for the future of cancer.

Another novel microRNA, miR-155 was shown to be elevated in serum of breast cancer (Liu *et al.*, 2013). This study analyzed miR-155 in serum of breast cancer patients and showed that it was up regulated and this elevation was correlated with clinical stage and p53 status. The study recommended that the efficient extraction and accurate identification of miRNAs from serum represent the first step to identify blood based detection test for breast cancer.

2.1.2.10 MicroRNA and apoptotic genes:

Cancer cells will change everything inside the cells and have the ability to alter the balance between pro and anti apoptotic factors to allow cell survival even in the presence of strong apoptotic inducer (Alberts *et al*., 2002). Apoptosis is a programmed physiological mode of cell death. De-regulation of apoptosis is a critical step in cancer, as it allows the genetically unstable cells to survive and accumulate further mutations that will leads to tumor formation (tumorigenesis) (Alberts *et al*., 2002).

Apoptosis can be initiated by death signals sent into the cell from other surrounded cells and by stress signals generated within the cell. Signals sent by other cells instructing a cell to undergo apoptosis. Apoptosis signaling is sent in response to conditions such as DNA damage in the nucleus, unfolded protein stress in endoplasmic reticulum or oxidative stress in the mitochondria (Meier *et al*., 2000).

The first paper published on miRNA role in apoptosis of human tissue was by Cimmino *et al*., (2005) describe how miR-15a and miR-16-1 expression inversely correlate with BCL2 expression. BCL2 protein is an anti apoptotic family member that inhibits Bax. While by inhibiting BCL2 expression, Bax will be continually activate and induce the intrinsic pathway of apoptosis.

BCL2 didn't behave like a typical oncogene; instead of disrupting normal proliferation controls, it promotes cell survival by blocking programmed cell death (Hockenbery *et al*., 1990). BCL2 function as sensors and regulators of apoptosis program.

Bax is a death promoter and have been shown in different studies to be in a mutated form in different tumors (Wallace- Brodeur and Lowe, 1999). The disruption of this gene in a study of Shibata and his coworker 1999, shown to accelerate brain and mammary tumorigenesis in a study carried in transgenic mice

MiRNA-21 was frequently shown in different studies to be over expressed in many cancers including; glioblastoma, colorectal, lung, breast and pancreatic cancers (Zhang *et al*., 2008a; Wei *et al*., 2011). Down regulation of miRNA-21 in breast cancer inhibited both tumor growth in vivo and cell growth in vitro, as a result of increased expression of its target BCL2 (Zhu *et al*., 2008).

P53 is a tumor suppressor protein that plays role in senescence, apoptosis, cell cycle arrest, DNA damage and other cellular response. MiRNA-34 family including miR-34a, miR-34b and miR-34c all show to be downregulated in several types of cancers and are direct transcription target of p53 (Zenz *et al*., 2009; Lee *et al*., 2011). Expression of miR-34 induces cell cycle arrest and leads to apoptosis or cellular senescence, whereas reduction of miR-34 function attenuate p53 mediated cell death (He *et al.*, 2007a).

P21 is one of the most important proteins in the p53 pathway, acting immediately downstream and mediating many of the actions of p53 (Zhao *et al*., 2000). Study of Biggs and Kraft, (1995) show that p21 may be rarely mutated in human cancer. MiRNA -106b has been shown in the study of Ivanovska *et al.*, (2008) to regulate p21 check point either promote cell cycle progression or cause cells to accumulate in G1 phase.

TWIST is a class II member of the basic helix-loop-helix transcription factor family. Its role in embryonic development and in carcinogenesis has been determined. Expression of TWIST is activated in different cancers including; breast, gastric, prostate, hepatocellular and bladder cancers. TWIST has been studied to be up regulated in cancers and usually correlated high levels of aggressiveness and poor patient survival rates (Qin *et al*., 2012).

TWIST expression may increase subpopulation in breast cancer stem like cells in culture, which may be relevant to drug resistance and distant metastasis (Micalizzi *et al.*, 2010).

A recent study proved that TWIST expression inversely correlated with ER-alpha expression in breast cancer cell lines and human breast invasive ductal carcinoma (Fu *et al.*, 2012).

2.1.2.10 General view of the method using TaqMan® qPCR,

Many methods are available for microRNA expression measuring but each has its own advantage and disadvantages. Quantitative PCR, microarray, northern blots, *in situ* hybridization and deep sequencing. Microarray and qPCR are the most two methods used for miRNA expression, however a study carried by Watson and Witwer, (2012) suggests that microarray may not directly give the result about the level of miRNA expression.

Following RNA extraction and purification, cDNA synthesis and subsequent miRNA profiling will be carried out by real time PCR method. The specificity and sensitivity of this method let almost all the studies to many challenges have been summarized in the review of Kang *et al.*, (2012) for using this method to analyze miRNA profiling due to the following reasons: first, miRNA length, its length is too short to provide sequence from primer design; second, miRNAs are highly conserved in sequence; third, there are more than one form of miRNA for example, primary miRNA (pri-miRNA), precursor miRNA (pre-miRNA) and mature miRNA, therefore, requires high specificity to recognize the mature miRNA.

So there are two qPCR methods for miRNA expression: the stem loop method and the poly (A) method. It is better to start talking about the first method the stem loop method, uses pre designed stem loop reverse

transcription RT primers for synthesizing cDNA of miRNAs which is as suggested to be the most extensively used method (Stratagene, 2004).

The stem loop RT primers is an oligonucleotide that forms a stem loop structure containing a universal reverse primer sequence on the loop and several (usually six) specific bases at 3' end that are complementary to the 3' end of the specific mature miRNA as shown in details in figure (2-9). After RT, the cDNA of miRNA can be quantitatively amplified with specific forward and universal reverse primers (Kang *et al*., 2012).

A dual labeled hydrolytic TaqMan probe that is specific for each miRNA is used in this method to increase miRNA specificity detection.
While the second poly (A) method using poly (A) tail that will be added to the 3' end of each mature miRNA by the assistant of poly (A) polymerase. Tailed miRNAs are then converted to cDNA using a universal RT primer containing 2-3 nucleotides at the 3' end followed by oligo dt and universal reverse primer sequence (Kang *et al*., 2012). This synthesized cDNA is then amplified with specific forward and universal primers (Stratagene, 2004). Here instead of using hydrolytic probe TaqMan, SYBR green I is used to quantitatively detect the amplified products and provides a more cost- effective detection method compared to using TaqMan probe method (Kang *et al*., 2012).

The disadvantage of using SYBR Green I dye is that it binds to all double stranded DNA including amplification products of target miRNA, contaminated genomic DNA and primer dimer, for that poly (A) method is not specific as stem loop method (Kang *et al*., 2012).

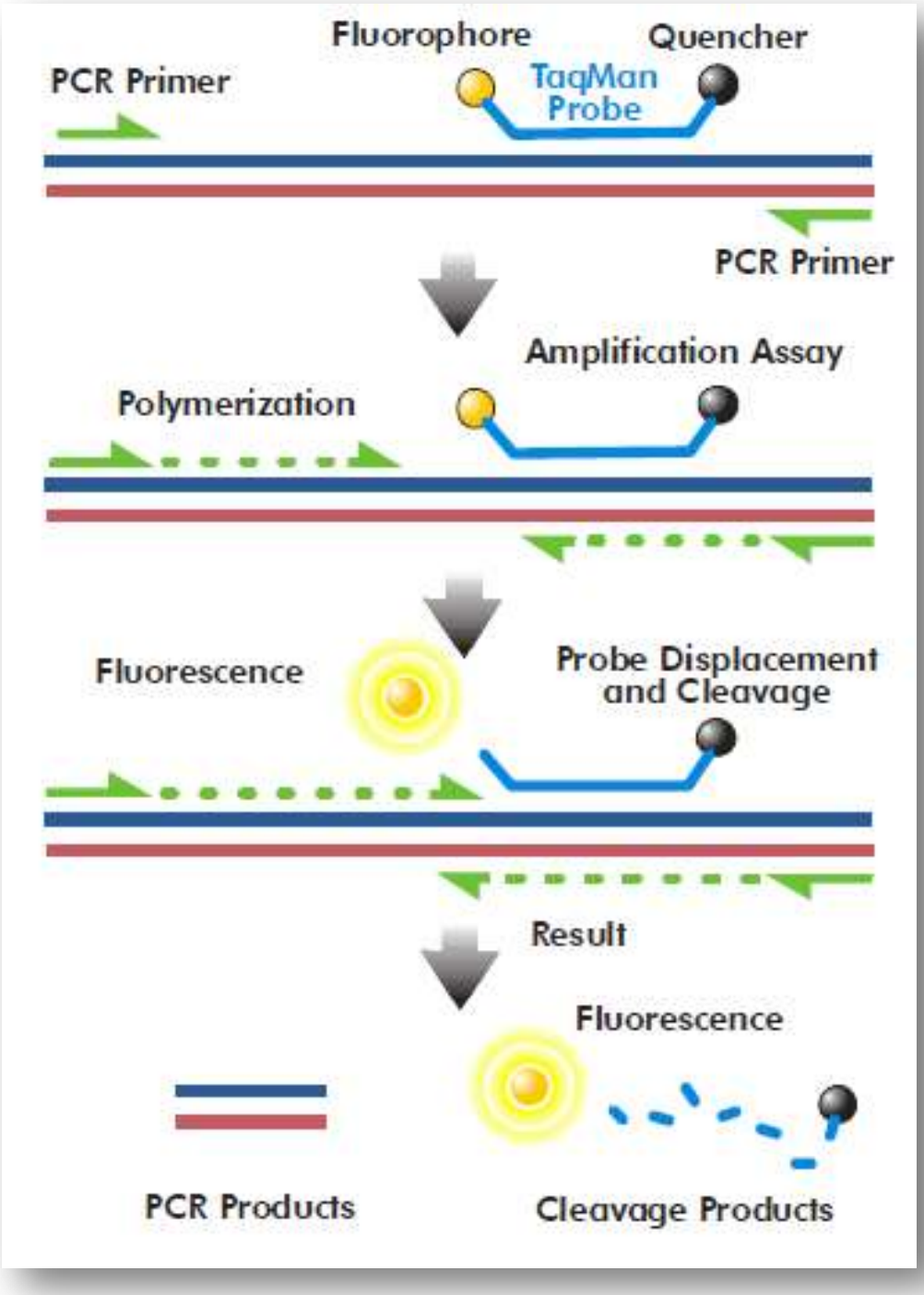


Figure (2-9): TaqMan probe chemistry mechanism of its work, (Kang *et al*., 2012)

2.1.2.11 Using Cobas-e-411 for hormone calculation levels and CA15-3

Roche Company at 2009 introduced Cobas for diagnostic and monitoring applications of the professional laboratories. It represents the second generation of ECL technology. ECL means electrochemical luminescence, where E refers to electrical stimulation; C refers to chemical reaction and L to produces light. This innovative technology provides superior analytical performance. Increased in sensitivity means that extremely low levels of antigens, as well as subtle changes in levels can be detected using Cobas. The very wide measuring range facilitates cost and time efficient testing by reducing the need to dilute and repeat samples. www.roche.com.

Cobas system was used for these tests:

- Serum work area with clinical chemistry and immunochemistry
- Data management and pre analytical solutions
- Products for coagulation analysis and urine analysis
- Instrument for rapid blood and cardiovascular testing
- Polymerase chain reaction- based applications for virology and women's health testing

Cobas-e-411 easy to operate, requires less training, promotes staff flexibility and is designed to reduce complexity of laboratory operation and provides efficient and compatible solutions. Its capacity of 18 tests and through put of up to 88 tests per hour. www.roche.com

3. Materials and Methods

3.1. Materials

3.1.1. Equipment

Equipment and apparatus used in this study are mentioned in table (3-1).

Table (3-1): Equipment used in this study

Equipment	Company	Origin
Centrifuge (Minispin)	Technico Mini	Korea
Cobas e-411	Roche	Germany
Homogenizer	IKA T10 BASIC / ULTRA- TURRAX	UK
Hood	UV 4 PCR	Germany
Liquid nitrogen -196℃	Operon / UKAS	UK
Magnetic stirrer	Stuart /heat –stir /SB 162	UK
Microcentrifuge	Thermo scientific Heraeous Fersco 1017	UK
NanoDrop™1000 Spectrophotometer	NanoDrop Technologies	USA
Micropipettes	Discovery comfort	UK
Real time PCR machine	MX 3000P™ / Stratagene	USA
Refrigerator	LEC savawatt control	UK
Thermo cycler	Thermo electron corporation	UK

Vortex	Fine vortex: fine pcr	Korea
Eppendorf tubes	Eppendorfes UK Ltd	UK
QPCR plates	Appleton woods	UK

3.1.2. Chemicals and Kits

Chemicals and kits used in this study were presented in details in table (3-2):

Table (3-2): Chemicals and kits used in this study

Chemicals and kits	Company	Origin
Absolute ethanol	Scharlau	European Union
Apoptotic primers	Invitrogen/ life technologies	USA
cDNA kit preparation from total RNA	PrecisioPrecision picoScript™ Reverse Transcription kit/ Primer Design Ltd.	UK
Master mix	Primer Design	UK
MicroRNA extraction kit from tissue samples	Invitrogen/ life technologies	USA
Plasma/ Serum circulating RNA Purification Kit (slurry format)	NORGEN biotek corp.	Canada
SYBR green	Precision™ 2XqPCR	UK

TaqMan MicroRNA Reverse Transcription	Applied Biosystem	USA
TaqMan microRNA assay	Applied Biosystem	USA
Total RNA extraction from tissue samples	E.Z.N.A^{TM} total RNA kit I from OMEGA	USA

3.1.3. Kits used in this study:

3.1.3.1. MicroRNA extraction kit from serum and plasma samples

Plasma/Serum Circulating RNA Purification Kit (Slurry Format) (Norgen Biotek Corporation/ Canada), was used to isolate small molecules of circulating RNA. The components of this kit are:

1. PS Solution A
2. PS Solution B
3. PS Solution C
4. Wash Solution
5. RNA Elution Solution
6. Mini Filter Spin Columns
7. Collection Tubes
8. Elution tubes (1.7 ml)

3.1.3.2. MicroRNA extraction kit from tissue samples

This kit designed by Invetrogen^{TM} / life technologies/ USA and used for microRNA extraction from tissue samples, PureLink^{TM} microRNA Isolation Kit for efficient and rapid purification of small RNA. Kit components were as follows:

1. Binding Buffer L3

2. Wash Buffer W5
3. Buffer W4 for single column purification
4. Sterile, RNase- free water
5. Spin cartridges with Collection tubes
6. Wash tubes (2ml)
7. Recovery tubes (1.7ml)

3.1.3.3. Total RNA extraction kit from tissue samples

Total RNA was extracted from breast border and cancer tissues using E.Z.N.A™ total RNA kit I from OMEGA/ USA, the following components were included in this kit:

1. HiBind® RNA Mini Columns
2. Collection tubes 2ml
3. TRK lysis buffer
4. RNA wash buffer I
5. RNA wash buffer II
6. DEPC water

3.1.3.4. Kit for synthesis of cDNA from microRNA

TaqMan MicroRNA reverse transcription kit from Applied Biosystem/ USA, was used to convert extracted microRNA to cDNA with high specificity, the following components were included in the kit:

1. dNTPs mix (100mM)
2. Multiscribe reverse transcriptase enzyme (50U/µl)
3. Reverse transcription buffer (10x)
4. RNase Inhibitor (20U/µl)
5. Reverse transcription primer (5x)

3.1.3.5. Kit for synthesis of cDNA from Total RNA

Total RNA were extracted from tissue samples and used for synthesis for cDNA synthesis using 3 pg – 3 ng of RNA, PrecisioPrecision picoScript™ Reverse Transcription/ BRITISH, the following components were included in this kit:

1. Oligo -dT primer 60 µl (yellow)
2. Random nonamer primer 60 µl (red)
3. dNTP mix (10mM of each) 60 µl (orange)
4. DTT 100mM (black)
5. PicoScript enzyme (white)
6. PicoScript 10X reaction buffer (0.5ml) (white)
7. RNase/DNase free water (white)

3.1.3.6. TaqMan® microRNA qPCR kit

TaqMan MicroRNA array qPCR kit from Applied Biosystems / Life technologies/ USA, was used to perform amplification of specific microRNA primer used and kit components were:

1. TaqMan PCR Master Mix (2X)
2. TaqMan microRNA assay (20X) which contain sequences of microRNA primers that taken from miRBASE website. The selected primers were designed according to the commonly known miRNAs, while other primers were designed according to the types of microRNA expressed in cell lines or tissues. The selected microRNAs were: let7a, miR-21, miR-15b, miR-26b, miR-27a, miR-34a, miR-34b, miR-429, mir-205, miR-218, miR-222, miR-378 and miR-191 (as endogenous control).

3.1.3.7. Total RNA qPCR kit

This kit was purchased from Primer Design Company/ UK which contains master mix reaction buffer including SYBR green dye.

3.1.3.8. Apoptotic genes primers sequences:

Primers for the apoptotic genes were purchased from Invitrogen / Life Technology Company/ USA. Sequences of these primers were present in table (3-3).

Table (3-3): Apoptotic genes primers

Apoptotic gene	Sequence (5'— 3')	Size (bp)	Tm (℃)
BCL2	F: TGGGATGCCTTTGTGGAACT	20	47
	R: GAGACAGCCAGGAGAAATCAAAC	23	50
p53	F: AGAGTCTATAGGCCCACCCC	20	51
	R: GCTCGACGCTAGGATCTGAC	20	51
P21	F: AAGACCATGTGGACCTGT	18	43
	R: GGTTGAAATCTGTCATGCTG	20	45
BAX	F: CTTTTGCTTCAGGGTTTCATCC	22	48
	R: TTGAGACACTCGCTCAGCTTCT	22	50
TWIST	F: GCCAATCAGCCACTGAAAGC	20	49
	R: TGTTCTTATAGTTCCTCTGATTGTTACCA	29	51
BRCA2	F: AATGCCCCATCGATTGGTC	19	46
	R: AGCCCCTAAACCCCACTTCAT	21	49

3.1.3.9. Estrogen hormone kit

Estrogen hormone was determined in serum of Iraqi samples collected from healthy women and women with breast cancer. This test was carried at Al-Nadaer Clinical Laboratory/ Baghdad using kit with the following components:

1. Container M: Streptavidin- coated microparticales (0.72mg/ml)
2. Container R1: Biotinylated polyclonal anti-estrodiol antibody (rabbit) (45 ng/ml) + Mesterolon (130 ng/ml)
3. Container R2: Estrodiol derivative- labeled with ruthenium complex (1.75 ng/ml)

3.1.3.10. Progesterone hormone kit

Progesterone hormone was determined in serum of Iraqi samples collected from healthy women and women with breast cancer. This test was carried at Al-Nadaer Clinical Laboratory/ Baghdad using kit with the following components:

1. Container M: Streptavidin- coated microparticales (0.72mg/ml)
2. Container R1: Biotinylated monoclonal anti-progesterone antibody (mouse) (0.15 mg/ml)
3. Container R2: Progesterone (of vegetable origin) coupled to synthetic peptide labeled with ruthenium complex (10 ng/ml)

3.1.3.11. Cancer Antigen (CA15-3) marker kit

Cancer Antigen (CA15-3) marker was determined in serum samples from Iraqi healthy women and women with breast cancer. This test was also achieved at Al-Nadaer Clinical Laboratory/ Baghdad, this kit was composed with following components:

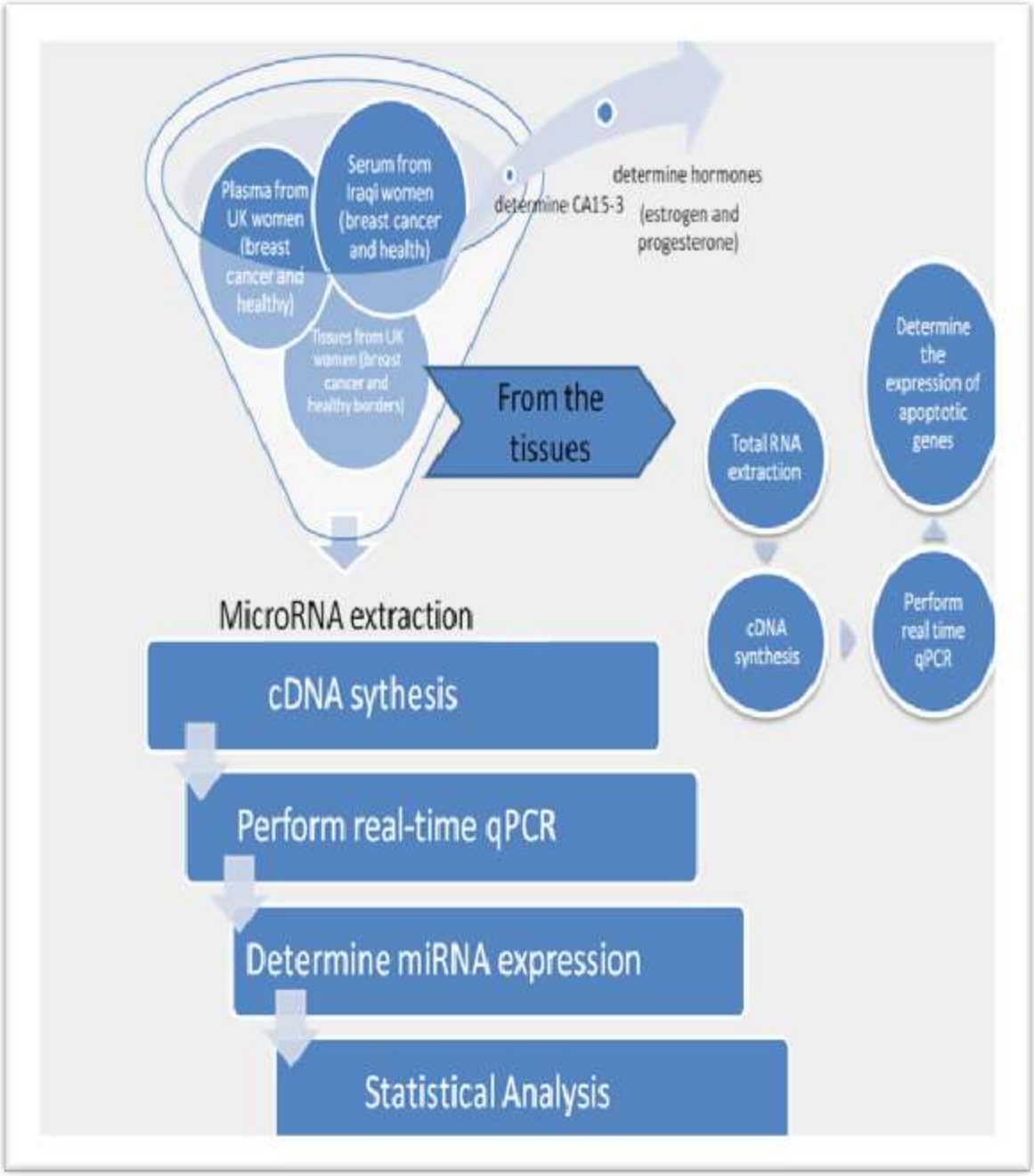
1. Container M: Streptavidin- coated microparticales (0.72mg/ml)
2. Container R1: Biotinylated monoclonal anti antibody (115D8mouse) (1.75 mg/L)
3. Container R2: monoclonal anti- CA15-3 anti body labeled with ruthenium complex (10 mg/L).

3.2. Methods

3.2.1. Subjects

3.2.1.1. Serum samples from Iraqi women

Blood samples were collected in 10 ml test tubes (test tubes special for serum separation). From total of twenty one serum samples including, six serum samples from healthy women volunteers and fifteen serum samples from women diagnosed with breast cancer. The blood then left for half an hour then centrifuged for 5minutes at 1600 rpm. Serum was transferred into 2ml eppendorf tubes and stored at -20 ℃ for further analysis. The main data and parameters included in this study were: women age, marital status, family history of breast cancer and others was shown in appendix I. These data were obtained from women attend Breast Cancer Unite/ Al- Karama Teaching Hospital/ Baghdad. Current study steps were determined in the scheme (3-1).



Scheme (3-1): Steps of the research project

3.2.1.2. Plasma samples from British women

Plasma samples were kindly supplied from Dr. James Brown, LHS lab. / Aston University/ UK and were frozen at -80 ℃ until use. These samples were previously collected from thirteen British women with breast cancer and eight healthy volunteer women.

3.2.1.3. Tissue samples from British women

Tissue samples were kindly supplied from Dr. James Brown, LHS lab. / Aston University/ UK and were frozen at -80 ℃. The tissues were previously taken from twelve matched British plasma breast cancer women and their twelve bordered tissues represent controls.

3.2.2. Detection of hormones and CA15-3 levels

Hormones levels (Estrogen and progesterone) and CA15-3 marker were detected in serum of blood samples from Iraqi healthy women and women with breast cancer. These tests were achieved for detection and measuring each hormone level and CA15-3 marker and analyzed by the Cobas e-411 instrument.

3.2.3. MicroRNA extraction from serum and plasma Samples

Plasma/ serum circulating RNA purification kit (slurry format) Norgene/ BIOTEK CORP/ Canada, was used for extraction of miRNA from Iraqi serum samples and British plasma samples. Kit components were mentioned in item (3.1.3.1), microRNA extraction was according to the following procedure:

1- To 1 ml of serum or plasma, a mixture of two solutions (1.8 ml lysis buffer solution B and 0.2 ml solution A) was added, vortexed for 15 seconds and incubate at 60°C for 10 min.

2- After incubation, 3ml of absolute ethanol was added. Mixed by vortexing for 15 seconds and centrifuged at 1000 X g for 30 seconds.
3- The supernatant was carefully decanted, then 0.3 ml of solution C was added to the slurry pellet, mixed by vortexing for 15 seconds and incubate at 60 °C for 10 min.
4- After incubation, 0.3 ml of absolute ethanol was added, mixed by vortexing for 15 seconds, then mixture was transferred to a mini filter spin column and centrifuged for 1 minute at 14000 X g. Then flow through was discarded.
5- Aliquot of 0.4 ml of washing solution was added to the column and centrifuged for 1 minute at 14000 X g. The flow through was discarded. This step was repeated three times
6- The empty column was centrifuged for 3 minutes at 14000 X g, and then collection tube was discarded.
7- Spin column was transferred to a fresh 1.7 ml elution tube, and 100 µl of elution solution was added to the column and centrifuged for 2 minutes at 2000 X g, followed by centrifugation for 3 minutes at 14000 X g.
8- Free circulating RNA was in the solution and stored at -80℃ until use.

3.2.4. MicroRNA extraction from British tissue Samples

Tissues samples from British women with breast cancer and healthy borders were collected during surgery and stored at -80℃ at LHS lab/ Aston University/ Brimingham/UK until use. MicroRNA was extracted using the procedure of the kit Invetrogen / life technologies/ USA and its components were mentioned in item (3.1.3.2). The protocol of microRNA extraction from tissue samples was described as follows

1- Aliquot of 300µl of binding buffer L3 was added to a homogenized piece of 5mg of minced tissue by using tissue homogenizer.

2- The mixture was centrifuged at 12000 X g for 1 minute to remove any particles. The supernatant was transferred to a new eppendorf and 300µl of 70% ethanol was added and mixed by vortexing.

3- The mixture was transferred to spin cartridge in a collection tube and centrifuged at 12000 X g for 1 minute. The flow through was kept.

4- Aliquot of 700µl of absolute ethanol was added to the flow through and mixed by vortexing.

5- The mixture was transferred to a second spin cartridge in collection tube and centrifuge at 12000 X g for 1 minute. Then the flow through was discarded.

6- To the spin cartridge, 500 µl of washing buffer W5 (after adding 40 µl of absolute ethanol to 10 ml wash buffer and mix) was added and centrifuged at 12000 X g for 1 minute. The flow through was discarded and this step was repeated once more.

7- The collection tube was removed and the spin cartridge was placed in a washing tube supplied with the kit. The spin cartridge was centrifuged at maximum speed for 1 minute to remove any remaining washing buffer.

8- The spin cartridge was placed in a clean supplied recovery tube.

9- MicroRNA was eluted with 50-100 µl sterile RNase- free water and incubated at room temperature for 1 minute.

10- The spin cartridge was centrifuged at maximum speed for 1 minute to elute the microRNA. The spin cartridge was discarded and the recovery tube contains purified microRNA and this was stored at -80℃ until use.

3.2.5. Estimation of RNA Concentration

The purity and concentrations of total RNA and microRNA were determined by measuring the absorbance of extracted RNA at 260nm (A_{260}) and 280nm (A_{280}) using Nanodrop™ 1000 spectrophotometer.

3.2.6. Reverse Transcription (RT) of microRNA

Reverse transcription was carried out using extracted microRNAs from serum, plasma and tissues samples. The reaction was performed by the TaqMan MicroRNA Reverse Transcription Kit, Applied Biosystems/ USA (item 3.1.3.4) as follows:

1. Reverse transcription master mix prepared by scaling the volumes of components to the desired number of RT reactions as in table (3-4).

Table (3-4): MicroRNA reverse transcription PCR mix components

Component	RT Master mix volume µl /15µl Reaction
dNTP mix (100mM)	0.15
Multiscribe RT enzyme (50U/µl)	1.00
10x RT buffer	1.5
RNase Inhibitor (20U/µl)	0.19
Nuclease free water	4.16

2. The components were mixed gently and centrifuged briefly.
3. For each 15 µl RT reaction, combine total RNA (1 - 10 ng of total RNA per 15 µl reaction) was added to the RT master mix in a ratio of 5 µl RNA: 7 µl RT master mix.
4. For a new eppendorf tube, 3µl of the RT primer was added.
5. The mixture was mixed gently and centrifuged briefly, then incubated on ice for 5 min.

6. The eppendorf tubes were placed carefully in the thermocycler and program for amplification was carried under the following conditions and start run:

Step	Time (minutes)	Temperature (℃)
Denaturation	30	16
Annealing	30	42
Extension	5	85
Hold	∞	4

7. At the end of amplification, the cDNA was stored at -20°C until used for quantitative real time-PCR.

3.2.7. Determine of microRNAs expression in samples using real-time qPCR

Real –time qPCR quantification of miRNAs expression in samples was performed using TaqMan® microRNA assays, Applied Biosystems/USA, its components were mentioned in item (3.1.3.6), and the following steps were carried out:

1- Master Mix was made by mixing the following components:

Component	Master mix volume/20µl reaction
TaqMan small RNA assay (20x)	1.00
Product from Rt reaction*	1.33
TaqMan universal pcr master mix (2x), no UNG	10.00
Nuclease free water	7.67

*The RT primer must be diluted a minimum of 1:15 in the final qpcr reaction.

2- The plate was sealed with the cap.

3- The program was setup as follows:

Step	Enzyme activation	PCR	
	Hold	Cycles (40)	
		Denature	Anneal/ extend
Temperature	95℃	95℃	60℃
Time	10 minutes	15 seconds	60 seconds

4- The program was run until end and the result was saved to perform the analysis.

3.2.8. Total RNA extraction from tissue samples

Breast cancer and normal tissues were obtained from British women undergo surgery and collected by Dr. James Brown/ Aston University and saved at LHS lab. at -80 ℃. The protocol of E.N.Z.A ™ total RNA kit I from OMEGA/USA was adopted to extract total RNA from these tissues to determine the expression of some apoptotic genes. Kit components were mentioned before in item (3.1.3.3.) using the following procedure:

1- A portion of 30 mg of breast tissue was minced using tissue homogenizer, and then 700 µl of lysis buffer TRK was added and mixed.
2- The mixture was centrifuged at 12000 X g for 2 minutes to remove any particles. Then the supernatant was transferred to a new eppendorf tube and equal volume of 70% ethanol was added to the lysate and mixed by vortexing.
3- The sample was transferred to HiBind RNA spin column in a collection tube and centrifuged at 12000 X g for 1 minute. The flow through was discarded.

4- From wash buffer (I) 500 µl was added to the HiBind RNA spin column and centrifuged at 12000 X g for 1 minute. The flow through was discarded.
5- The spin cartridge was washed with 500µl of washing buffer and centrifuged at 12000 X g for 30 seconds. The flow through was discarded.
6- Wash step was repeated once more.
7- The collection tube was discarded and the HiBind RNA spin column was placed in a wash tube supplied with the kit. The HiBind RNA spin column was centrifuged at maximum speed for 1 minute to remove any remaining washing buffer.
8- The HiBind RNA spin column was placed in a clean recovery tube.
9- Total RNA was eluted with 50-100 µl sterile DEPC water.
10- The spin cartridge was centrifuged at maximum speed for 2 minute. The recovery tube contains purified RNA. The spin cartridge was discarded and RNA containing tube was stored at -80℃ until use.

3.2.9. Conversion of total RNA into cDNA

Reverse transcription was performed using cDNA Reverse Transcription Kit from Primer Design and its components were mentioned in item (3.1.3.5.) and following procedure was performed:

1. RT master mix was prepared by scaling the volumes of components to the desired number of RT reactions:

Component	Master mix volume/10 µl reaction
RNA template (3 pg- 3 ng)	X µl
RT primer	1.00µl
Nuclease free water	X µl

2. The eppendorf tubes were incubated on hot plate and heated to 65℃ for 5 min.
3. Eppendorf tubes were immediately cooled in ice jar.
4. The master mix of Reverse transcription was prepared for the reaction as shown in table (3-5).

Table (3-5): Total RNA reverse transcription PCR mix components.

Component	Master mix volume (µl) /10µl reaction
dNTPs mix (10mM)	1.0
PicoScript RT enzyme	1.0
PicoScript 10x RT buffer	2.0
DTT	2.0
Nuclease free water	4.0

5. From this mixture 10 µl was added to each eppendorf tubes on ice and mixed by vortexing
6. For specific gene amplification thermocycle incubation was run for the following cycles:
   - First at 55℃ for 20 min
   - Second at 75℃ for 15 min
7. At the end, cDNA was stored at -20℃ until use.

3.2.10. Real time qPCR preparation from cDNA to determine the expression of the apoptotic genes

For the quantification and finding the expression of some selected apoptotic genes in tissues of borders and breast cancer British women, qPCR was performed using SYBR green Master Mix from Primer Design using Stratagen 3000x machine. B-Actin gene was used for normalization. The following components were mixed to perform real- time PCR reaction:

Component	Master mix volume (µl) /20µl reaction
SYBR green	10.0
Primers	1.0
cDNA	5.0
Nuclease free water	4.0

When all the components were added on real-time PCR plate, the program was run according to the following steps:

Step	**Enzyme activation**	**PCR**			**One cycle**		
	Hold	**Cycles (40)**					
		Denature	Anneal	Extend			
Temperature	95℃	95℃	60℃	70℃	95℃	55℃	95℃
Time	10 min.	15 sec.	60 sec.	60 sec.	30 sec.	30 sec.	30 sec.

3.2.11. Determination of miRNA and mRNA expressions

MiR-191 was found to be abundantly expressed in all samples analyzed in this study, in cancer and healthy serum, plasma and tissues alike, with very little variability between samples; accordingly it was chosen as an endogenous control. In the study of Hu *et al*., (2012) tested several microRNAs to determine the best endogenous control for the studies in circulating miRNAs; they proved that miR-16 was not suitable selection as endogenous control while miR-191and miR-484 may serve as good candidate. Small nuclear RNA U6, SNORD and rRNA 5S have been used previously in studies but their instability in blood and not detected in blood or have high variability between samples so it could not be suitable for normalization of extracellular miRNA (Wang *et al*., 2012).

The (ΔCt) values for samples were calculated using the mean expression value of all expressed miRNAs in a given sample the global mean (Mestdagh *et al*., 2009) with the following equation:

ΔCt = Ct (global mean) - Ct (assay)

The relative quantitative gene expression level was evaluated using the ΔΔCt comparative Ct method.

Fold inductions were calculated using the formula 2^ (ΔΔCt) where**:**

ΔCt=Ct (target gene)-Ct (reference gene)

ΔΔCt=ΔCt (patients) − ΔCt (control).

3.2.12 Statistical and data analysis

Student's t test was used to evaluate differential expression of microRNA between breast cancer and normal samples. P value less than < 0.05 was considered statistically significant. All the graph was plotted using GraphPad Prism6 software. For correlation between hormones and CA15-3 marker, Pearson statistical analysis was used.

4. Results and Discussion

4.1 Collection of Iraqi blood samples

Blood samples were collected from 15 Iraqi women diagnosed with breast cancer and from six healthy women represent control group. Results in table (4-1) indicate that the age of women was ranged between 32-73 years, with average age of 49 years of breast cancer women while the average age of healthy women was 41 years. Other history parameters of patients and healthy women are mentioned in table (4-1).

Serum of blood samples was obtained from these cases to determine estrogen hormone, progesterone hormone, CA15-3 marker and expression of microRNAs.

4.2 Hormones levels and CA15-3 marker in Iraqi women with breast cancer and healthy women

Estrogen, progesterone hormones and CA15-3 marker were measured in Iraqi serum of breast cancer diagnosed women and healthy women using Cobas-e-411analyzer. All the parameters were measured at the same time in all women (estrogen, progesterone and CA15-3 levels).

Results indicated in table (4-2), showed that there was a significant correlation between the level of estrogen hormone and age of healthy women (r^2= 0.9), while an inverse significant correlation between the level of progesterone hormone and age of healthy women (r^2=¯0.9) and a significant correlation between estrogen hormone and CA15-3 marker in healthy women group (r^2= 0.6).

Table (4-1): Hormones level and CA15-3 status in serum of Iraqi women

Case	Sample	Age (year)	Estrogen (pg/ml)	Progesterone (ng/ml)	CA 15-3 (U/ml)	BIRADS * result
1	Normal	63	15.51	0.356	13.18	1
2	Normal	64	17.69	0.514	None	1
3	Normal	42	155.3	0.31	16.48	2
4	Normal	40	46.35	0.392	6.10	2
5	Normal	40	38.28	0.263	14.31	1
6	Normal	22	128..9	0.955	13.77	None
7	Cancer	50	25.29	0.324	9.97	4
8	Cancer	56	18.92	0.077	70.65	Metastasis
9	Cancer	63	32.3	0.323	9.93	Ductal Carcinoma
10	Cancer	47	205.3	0.434	15.41	4
11	Cancer	50	248	0.226	14.26	3
12	Cancer	36	130.9	6.03	22.22	4
13	Cancer	45	119.7	0.193	4.89	4
14	Cancer	50	188.2	0.06	19.89	4
15	Cancer	50	22.6	0.599	14.76	3
16	Cancer	46	27.26	0.181	3.72	None
17	Cancer	36	101.5	0.302	18.66	Zero
18	Cancer	50	23.85	0.402	None	4
19	Cancer	73	14.88	0.436	23.94	3
20	Cancer	32	220	9.5	18.97	4
21	Cancer	54	25.98	0.176	29.9	5

*BIRADS: Breast Imaging-Reporting and Data System**.** BI-RADS Assessment Categories are: 0: Incomplete, 1: Negative, 2: Benign finding(s), 3: Probably benign, 4: Suspicious abnormality, 5: Highly suggestive of malignancy, 6: Known biopsy – proven malignancy. (American College of Radiology, 2003)

Table (4-2): Correlation between estrogen, progesterone, CA15-3 and age in healthy women in current study

Factors	Age	CA15-3	Progesterone
Estrogen	**0.9**	**0.6**	**−0.09**
Progesterone	**−0.9**	**0.02**	
CA15-3	**−0.09**		

In breast cancer women the data presented in table (4-3); showed an inverse significant correlation between (estrogen hormone, progesterone hormone and CA15-3) and age (r^2=¯0.4, 0.2 and 0.03) respectively, predicting that estrogen hormone decrease with age whereas progesterone levels increased with age in breast cancer women and without any indication of importance of CA15-3 marker in breast cancer women age.

Table (4-3): Correlation between estrogen, progesterone, CA15-3 and age in breast cancer women in current study

Factors	Age	CA15-3	Progesterone
Estrogen	**−0.4**	**0.2**	**−0.2**
Progesterone	**0.2**	**−0.3**	
CA15-3	**0.03**		

The associations between estrogen and progesterone levels in premenopausal women and breast cancer risk have not been consistent, and further assessments are needed as suggested by the study of Fortner *et al*., (2013). The positive associations between endogenous estrogens and androgens and postmenopausal breast cancer risk are well established (Falk *et al*., 2013). The complexity of measuring estrogens in different

phases of the menstrual cycle may have contributed to the inconsistencies in the literature. In study to detect a significant association with estradiol, follicular, but not luteal, levels were associated with risk, thus further assessments stratified by menstrual cycle phase are needed (Falk, *et al*., 2012; Fortner *et al*., 2013).

Estrogen hormone was found to contribute to tumors by promoting the proliferation of cells with existing mutation or perhaps by increasing the opportunity for mutations that regulate the growth and differentiation of mammary cells which may play an important role in the development of breast cancer (Henderson and Feigelson, 2000).

Progesterone has been shown that it either decreases the incidence of breast cancer by migrating estrogen- induced proliferation in breast epithelial cells or increases the opportunity in cancer development due to high breast cell proliferation in the luteal phase when progesterone levels increased (Doisneau-Sixou *et al*., 2003). Endogenous progesterone was not statistically associated with breast cancer as suggested by the study of Schernhammer *et al*., (2013).

There are two scenarios regarding the hormonal profile and its relation to breast cancer hormones toward breast cancer and subsequently can be used as potential diagnostic biomarkers for early prediction of breast cancer and hormones away from breast cancer and frequently can be used as powerful and effective treatments as well as prognostic tools for breast cancer as presented by Ahmed and Abd-Rabou, (2013).

Cancer Antigen (CA 15-3) was found to be expressed at the luminal surface of most secretary epithelial cells and associated with breast tumors (Taylor *et al*., 2002). Cancer Antigen (CA 15-3) and CEA are the most common serum markers used for monitoring breast cancer. Due to their low specificity and sensitivity as indicated in figure (4-3),

they have no chance in breast cancer early detection (Park *et al*., 2008). The study of Tarhan and colleagues (2013), CA15-3 level was detected in the majority of the patients and was above the normal range at the time when metastasis was detected, but at the end of study it was concluded that CA15-3 level which is still not recommended by guidelines in routine practice.

4-3 Expression of microRNA

4-3-1 Expression of microRNA in Iraqi serum samples

4-3-1-1 Expression of let-7a

In this study, the expressions of let-7a was examined and shown to be over- expressed in serum of Iraqi patients with breast cancer women as presented in figure (4-1). The expression of let-7a in serum of breast cancer women was significant as compared with serum from healthy women volunteers with P= 0.0221. All results mean±SD determined in details in appendix II.

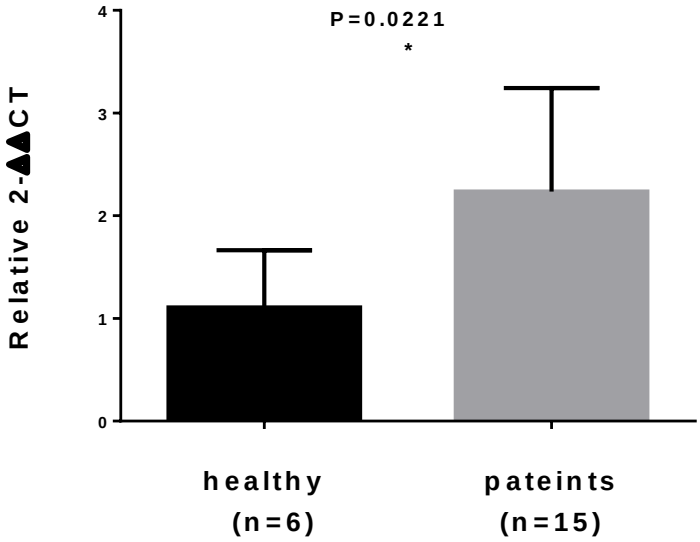


Figure (4-1): Expression of let-7a in serum of Iraqi women with breast cancer and healthy women volunteers, (*): means significant

As mentioned in studies, let-7a is involved in the cell proliferation and influence cancer metastasis in various tumors including breast cancer (Akao *et al*., 2006; Zhang *et al*., 2010a). Let-7a is one of the well – established cancer associated miRNAs and Yu *et al*., (2007) reported that let-7a was significantly elevated or increased in almost all of cancer patients tested such as lung, breast, colon, gastric, ovarian cancers.

In the circulation, Heneghan *et al*., (2010d) examined a panel of 7 candidate microRNAs (miR-10b, miR-21, miR-145, miR-155, miR-195, miR- 16 and let-7a) in the whole blood of breast cancer patients. They proved that miR-195 and let-7a levels were significantly higher in cancer patients than in controls.

A recent study mentioned that serum level of let-7a has no significant differences between gastric cancer patients and healthy. The study also found a positive correlation between serum miR-20a-5p and let-7a levels with serum pepsinogen A (PGA) and C (PGC) (Xu *et al*., 2013). They presented a good suggesting that these microRNAs give an aid in early diagnosis of cancers.

4-3-1-2 Expression of miR-21

MicroRNA-21expression level determined and shown to be elevated in serum of Iraqi breast cancer as presented in figure (4-2), this over-expression in miR-21 level in serum of breast cancer women was significantly different with healthy women volunteers with P= 0.0265. All results mean±SD determined in details in appendix II.

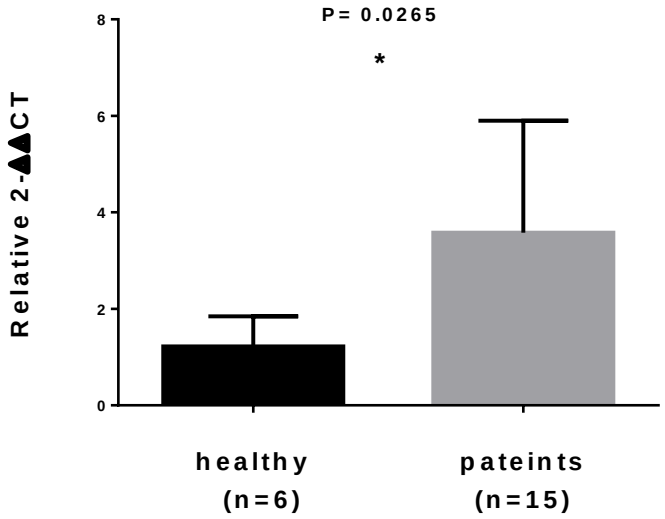


Figure (4-2): Expression of miR-21 in serum of Iraqi women with breast cancer and healthy women volunteers, (*): means significant

MicroRNA-21 has been described to be as an oncomiR and is found to be up-regulated in many solid tumors including breast cancer and hematological cancers (Yan *et al*., 2008; Yaman *et al*., 2011).

In relation to breast cancer serum miR-21 significantly increased in breast cancer especially in stage IV cancer (Wang *et al*., 2010a; Asega *et al*., 2011). The level of miR-21 in breast cancer serum was higher than in normal controls, especially in patients with lymph node metastasis as presented in the work of Ozgun *et al*., (2013).

In fact, the miR-21 gene is located on chromosome 17, which is located within the common fragile site FRA17B (Calin *et al*., 2004).This region is frequently found amplified in breast, colon, and lung cancer, consistent with the fact that miR-21 over-expression is widespread in many types of cancer, including the breast (Calin and Corce, 2007).

In a recent study of Si *et al*., (2013), it was found that miR-21 significantly up-regulated in breast cancer tissue and serum and this results indicated that miR-21 high expression associated with poor patient

survival concluded that this miRNA represent be a good early biomarker for cancer diagnosis and prognosis.

4-3-1-3 Expression of miR-27a

The expression level of miR-27a was determined in serum of Iraqi women as presented in figure (4-3). Serum of breast cancer patients showed high expression of miR-27a without significant differences between the cancer patients and normal women. This expression of miR-27a has p= 0.5764.

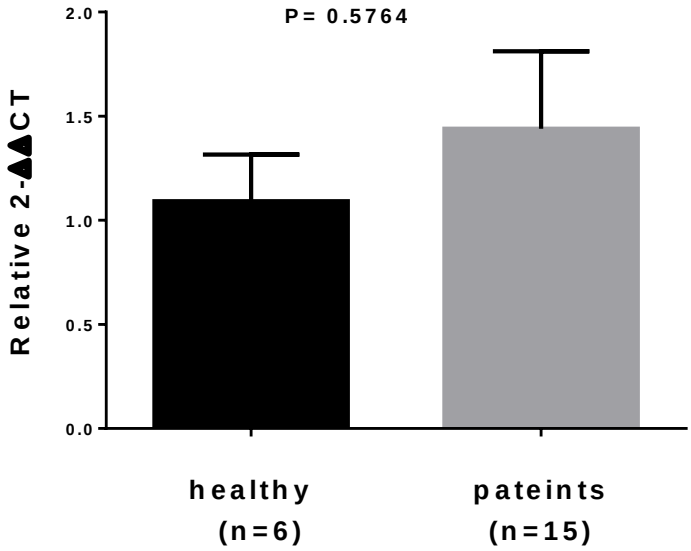


Figure (4-3): Expression of miR-27a in serum of Iraqi women with breast cancer and healthy women volunteers

MicroRNA-27a was reported as breast cancer oncomiR and found to be over-expressed in breast cancer cells (Mertens-Talcott *et al*., 2007).

It has been concluded that miR-27a promotes angiogenesis by mediating endothelial differentiation of breast cancer stem like cells. This finding highlights the use of microRNAs as a new target for anti-angiogenesis cancer therapy (Tang *et al*., 2013).

MicroRNA-27a was widely expressed in cancer cell lines and tends to be higher in breast cancer cell lines studied like (MDA-MB-231, MDA-MB-435, SKBR3 and MCF7) (Mertens-Talcott *et al*., 2007).

Next-generation Solexa sequencing results demonstrated that 19 serum miRNAs were markedly up-regulated in gastric cancer (GC) patients compared to the controls, and a profile of five serum miRNAs (miR-1, miR-20a, miR-27a, miR-34 and miR-423-5p) was identified as a biomarker for GC detection. The analysis results showed that the serum miRNA-based biomarker could also indicate the progression stages of tumors (Liu *et al*., 2011a).

4-3-1-4 Expression of miR-222

Expression of miR-222 in the serum of breast cancer patients compared to control healthy women was presented in figure (4-4). This expression of miR-222 was not significant in serum of breast cancer patients as compared to healthy women with P= 0.2341.

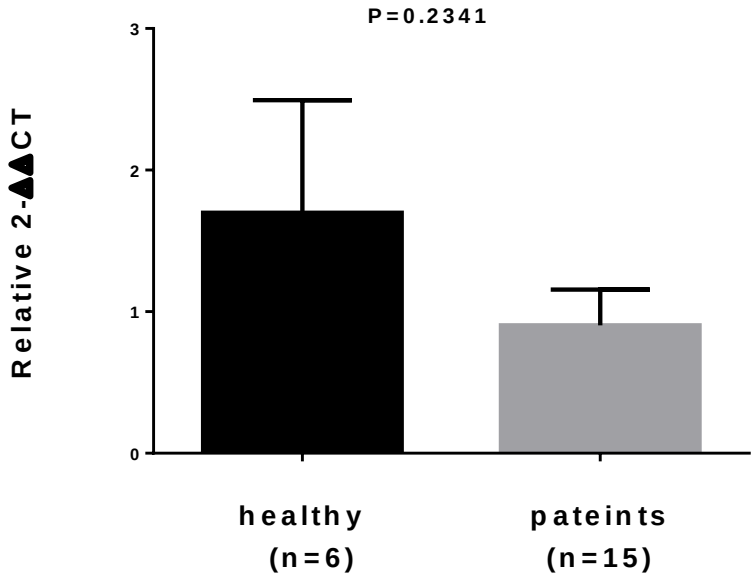


Figure (4-4): Expression of miR-222 in serum of Iraqi women with breast cancer and healthy women volunteers

Several miRNAs were mentioned in the study of Chen *et al*., (2013) such as miR-134, miR-146a, miR-221, miR-222 and miR-23a were significantly deregulated in the sera of colorectal and lung cancer patient groups, suggesting that a miRNA profile represent a good diagnostic tool for cancer.

MiR-222 was shown to promote cell growth, cell cycle progression and invasion in different cancer types, *in vitro* and *in vivo* (Pineau *et al*., 2010; Zhang *et al*., 2010b).

Increased expression of miR221 and miR-222 facilitates transit from quiescence to proliferation by targeting p27 proteins (Medina *et al*., 2008).

A study reported an increased expression of miR221 and miR-222 represent a signature of tamoxifen resistance in breast cancer and breast cancer cell lines (Miller *et al*., 2008).

4-3-1-5 Expression of miR-205

In this study, Iraqi breast cancer women showed low expression of miR-205 expression in the serum compared to healthy women as presented in figure (4-5). This down- regulation of miR-205 expression in the serum of breast cancer women was not significantly compared with healthy women with p= 0.1010.

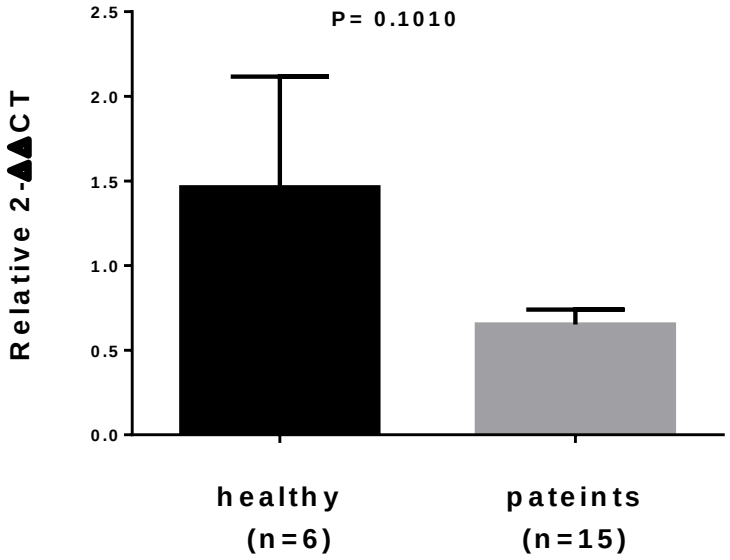


Figure (4-5): Expression of miR-205 in serum of Iraqi women with breast cancer and healthy women volunteers

Iorio *et al*., (2005) reported that miR-205 was down-regulated in breast cancer tumor tissues and also in metastatic breast cancer cell lines. Also Liu *et al*., (2013) found that expression of miR-205 in serum and tissue of breast cancer patients was down-regulated suggesting that this microRNA (miR-205) is a potential tumor suppressor in breast cancer. They also suggest that miR-205 suppresses proliferation and increases breast tumor cell proliferation.

MicroRNA-205 represents a tumor suppressor in human breast cancer cells by post transcription inhibition of Her2 re-expression. MiR-205 was down- regulated in these cancer cells and found to bind to 3'UTR region of Her2 receptor (Wang *et al*., 2013).

As for the biological function of miR-205, this microRNA cannot be clearly placed in the cancer development pathway due to its dual role, that it can be considered as an oncomiR or a tumor suppressor, depending on the cellular context and signaling pathway (Aushev *et al*., 2013).

4-3-1-6 Expression of miRNA-218

The expression of miR-218 level was up-regulated in serum breast cancer of Iraqi women compared to healthy women serum as showed in figure (4-6). This over- expression did not show any significant differences between breast cancer patient and normal women with p=0.1983 value. All results mean±SD determined in details in appendix II.

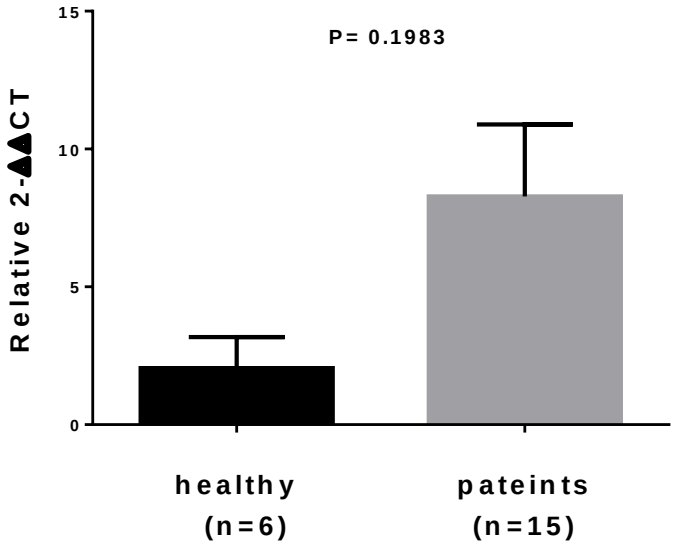


Figure (4-6): Expression of miR-218 in serum of Iraqi women with breast cancer and healthy women volunteers

It was known that the tumor suppressor activity of miR-218 is achieved through the inhibition of cancer- promoting genes such as mTOR-Akt signaling pathway (Uesugi *et al*., 2011) and MMP-9 pathway (Song *et al*., 2010a).

A study of Tatarano and his group (2011), demonstrated that miR-218 was down- regulated in breast cancer cell lines and found to decrease cell proliferation, migration and invasion activity and increase cell apoptosis in miR-218 tranfactant. This study suggested that miR-218 is a candidate tumor suppressor of breast cancer.

Down-regulation of miR-218 in invasive breast cancer cell lines have been reported in study of Yang *et al*., (2012) migration and invasion of breast cancer cell line was inhibited by re-expression of miR-218 in metastatic cells, which demonstrated that miR-218 act as a tumor suppressor.

4-3-1-7 Expression of miR-378

The expression of miR-378 was up- regulated as presented in this study in serum breast cancer of Iraqi women than healthy women as presented in figure (4-7). This over-expression in miR-378 between serums of breast cancer and healthy women was not significant difference with p= 0.5427. All results mean±SD determined in details in appendix II.

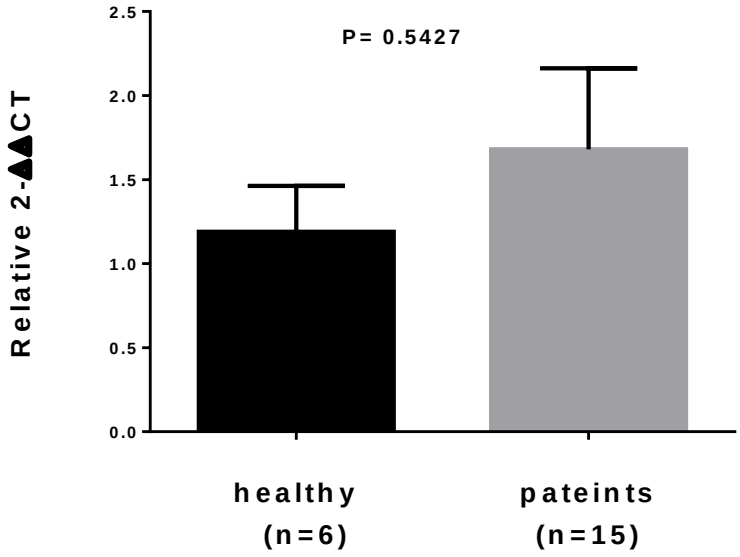


Figure (4-7): Expression of miR-378 in serum of Iraqi women with breast cancer and healthy women volunteers

Redova and colleagues (2012) found that miR-378 were up-regulated in serum of patients with renal cell carcinoma, suggesting that miR-378 represent a potent in discriminating RCC from healthy control

serum. Also up-regulation of miR-378 was detected in serum of gastric cancer patient compared to healthy group tested (Liu *et al*., 2012a). From these results it suggested that this micro-RNA could have a strong potential novel non- invasive biomarker in early diagnosis of cancers.

Array of microRNAs was used in study of Li *et al*., (2013a) to distinguish patients with pancreatic cancer from those normal one using their serum, the result of that study revealed that one of these microRNAs in the array miR-378 was elevated in serum of pancreatic cancer patients compared to control group.

4-3-1-8 Expression of miR-15b

Expression of miR-15b was detected in this study and it was up-regulated in serum breast cancer of Iraqi women compared to healthy women volunteer as presented in figure (4-8). This up-regulation in the expression of miR-15b in serum of breast cancer was not significantly different from serum of healthy normal women with p= 0.4592.

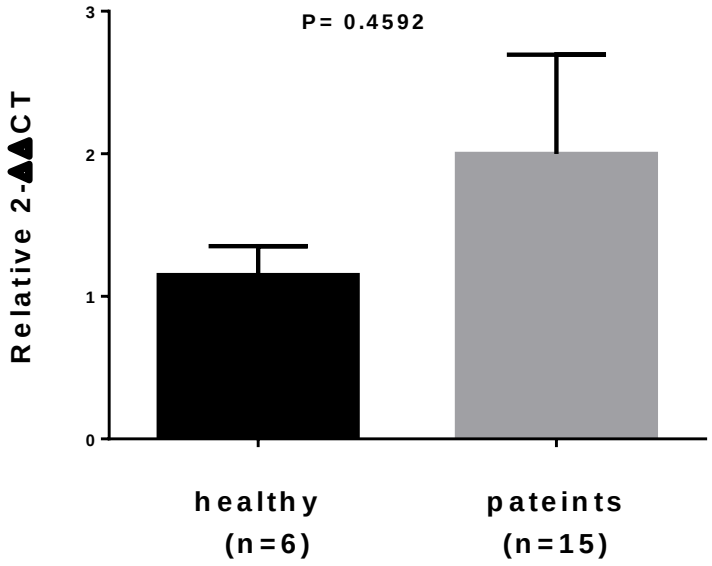


Figure (4-8): Expression of miR-15b in serum of Iraqi women with breast cancer and healthy women volunteers

The expression levels of miR-15b were shown in the study of Liu *et al*., (2012a) to be reduced in the serum after the surgery in hepatocellular carcinoma patients.

Also in recent study the level of miR-15b expression was determined in serum of fatty liver disease patients the expression was higher in patients compared to healthy volunteers (Zhang *et al*., 2013).

It was indicated in the study of Xia *et al*., (2009), that miR-15b might regulate cell cycle progression in glioma cells by targeting cell cycle regulated molecules; over- expression of miR-15b resulted in cell cycle arrest at the G0/G1 phase; while suppression of miR-15b expression resulted in decrease of cell population in G0/G1 and a corresponding in arrest of cell population in the S phase.

4-3-1-9 Expression of miR-26b

In figure (4-9), the expression of miR-26b was shown to be down-regulated in serum breast cancer of Iraqi women compared with normal healthy volunteers. This study show for the first time that the expression of miR-26b was down-regulated in serum breast cancer patients than healthy individuals as no pervious study investigated this expression. Thus the differential expression of miR-26b is novel finding in breast cancer early detection. The serum expression of miR-26b was statistically significant with p=0.0034. All results mean±SD determined in details in appendix II.

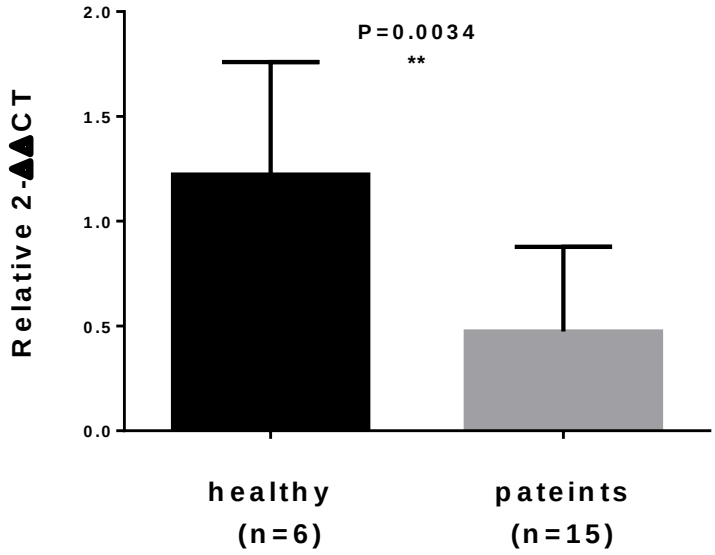


Figure (4-9): Expression of miR-26b in serum of Iraqi women with breast cancer and healthy women volunteers, (**): means significant

Several studies reported that miR-26b tend to be down-regulated in several cancers such as hepatocellular carcinoma (Ji *et al*., 2009a), nasopharyngeal carcinoma (Ji *et al*., 2010) and primary squamous cell lung carcinoma (Gao *et al*., 2011). While over-expression of miR-26b reported to induce apoptosis in MCF-7 breast cancer cell line (Liu *et al*., 2011).The study of Li *et al*., (2013) demonstrated that miR-26b inhibits the proliferation of MDA-MB-231 breast cancer cell lines and show that this miRNA was over-expressed in this cell line.

MicroRNA-26b was down-regulated in fibroblasts breast cancer as described in the study of Verghese *et al*., (2013). The study proved that reduced miR-26b expression caused to increase fibroblast migration and invasion.

4-3-1-10 Expression of miR-429

Significant novel finding was noticed in miR-429 expression in serum breast cancer of Iraqi women compared to serum of healthy women figure (4-10). This novel finding showed that expression of miR-429 was decreased in breast cancer patients compared with healthy serum women and give p= 0.0310. All results mean±SD determined in details in appendix II.

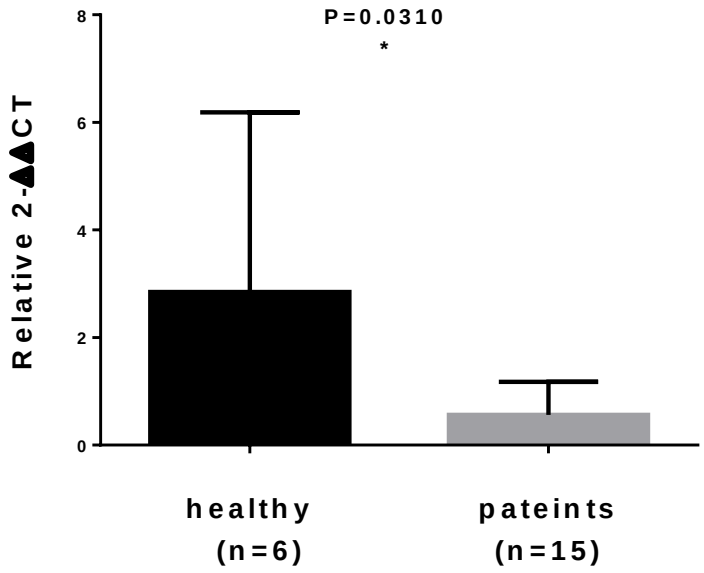


Figure (4-10): Expression of miR-429 in serum of Iraqi women with breast cancer and healthy women volunteers, (*): means significant

The expression of miR-200 family including (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) was presented to be lost in invasive breast cancer cell lines with a mesenchymal phenotype (Kastl *et al*., 2012). Recently, several studies mentioned that miR-429 expression may modulate the tumorgenesis of several types of cancers including, gastric (Sun *et al*., 2011), ovarian, pancreatic, breast and colorectal cancers (Li *et al*., 2013a; Hashimoto *et al*., 2013).

Down-regulation of miR-429 may be an important in late step in tumor progression and this dys-regulation of this miRNA expression can

modify tumor prognosis possibly through regulation of cell proliferation and apoptosis (Sun *et al*., 2011; Hashimoto *et al*., 2013).

4-3-1-11 Expression of miR-34a

As presented in figure (4-11), there are no significant differences in expression of miR-34a in serum of both breast cancer and healthy women. Even there is slight down regulation in breast cancer women but this down regulation was not significantly different from healthy women and has p= 0.544.

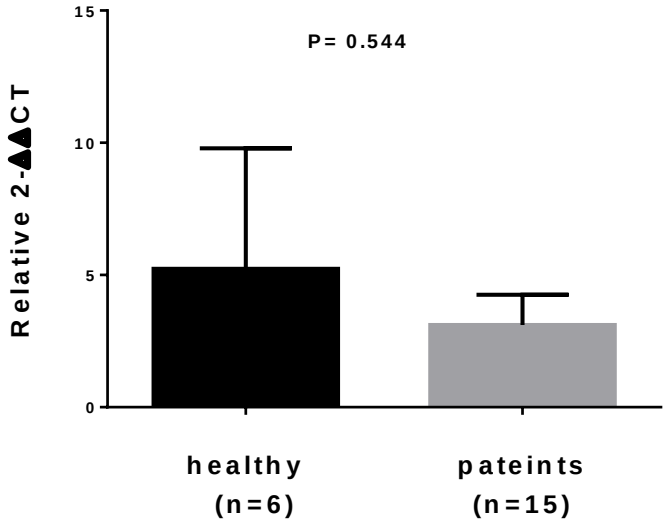


Figure (4-11): Expression of miR-34a in serum of Iraqi women with breast cancer and healthy women volunteers

MicroRNA-34a was reported to work as a tumor suppressor and regulates cell cycle, senescence, apoptosis and metabolism (Haigis and Guarente, 2006). Eichelser and partner (2013) reported that decrease in serum concentration of miR-34a of breast cancer patients tend to be significantly different between M0 breast cancer and healthy women. The study concluded the use of circulating miRNA as biomarker in the serum of breast cancer patients.

The study of Roth and coworker (2010) presented that the expression of miR-34a in serum of breast cancer patients was over-expressed compared with control group and this increasing was correlated with the occurrence of metastasis. While it was reported that miR-34a was downregulated in non-small cell lung carcinoma, pancreas tumor cell line, colon carcinoma and primary neuroblastomas (Bommer *et al*., 2007).

4-3-1-12 Expression of miR-34b

In this study, the expression of miR-34b in serum of Iraqi breast cancer and healthy women was determined and the result was shown in figure (4-12). There is no significant difference in the expression of miR-34b in breast cancer and healthy women, this expression has p=0.1132.

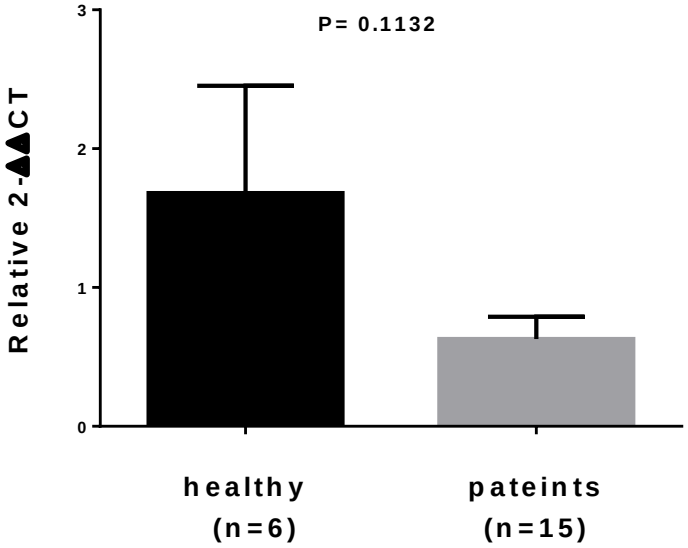


Figure (4-12): Expression of miR-34b in serum of Iraqi women with breast cancer and healthy women volunteers

The study of Lee *et al*., (2011) revealed that miR-34b is an onco-suppressor miRNA requiring both ER positive and wild type (wt) *p53* phenotype in breast cancer cells. Also in the same study they found that miR-34b inhibited breast cancer cell proliferation by targeting cyclin D1.

MicroRNA-34b expression found to be negatively correlated with disease free survival and overall survival in triple- negative breast cancer patients and suggested in study of Svoboda *et al*., (2012) to be a new promising prognostic biomarker in triple- negative breast cancer patients.

MicroRNA-34b and miR-34c expression is regulated by *p53*, which mediate cell cycle arrest and promotes apoptosis (He *et al*., 2007b). DNA damage or aberrant cell cycle progression leads to rapid increase in *p53* which in turn may leads to *p53*-dependent miR-34b expression (Corney *et al*., 2007).

4-3-2 Expression of microRNA in British plasma samples

4-3-2-1 Expression of let-7a

The expression of let-7a was determined in plasma of breast cancer women and healthy women of the British. Figure (4-13) showed significant up-regulation of let-7a in plasma of British breast cancer patients compared with healthy women. This over- expression of let-7a in plasma of breast cancer was significantly different compared with healthy women and has p= 0.0196. All results mean±SD determined in details in appendix II.

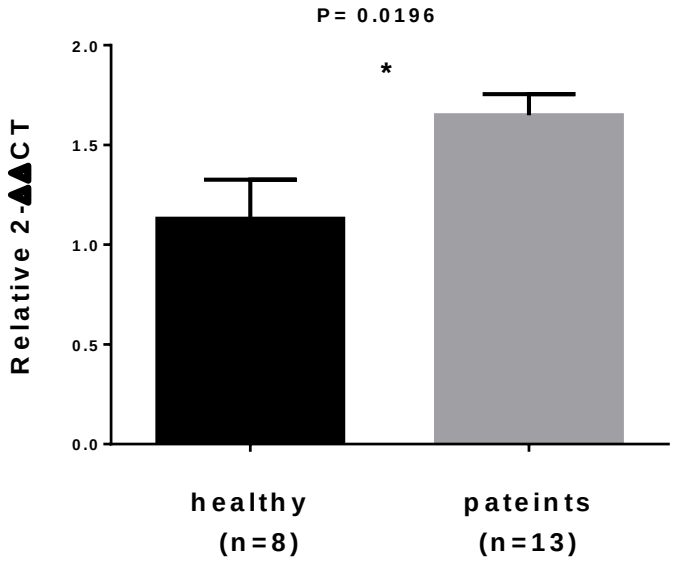


Figure (4-13): Expression of let-7a in plasma of British women with breast cancer and healthy women volunteers, (*): means significant

In breast cancer, it has been shown in study of Heneghan *et al*., (2010d) that let-7a was greatly increased in blood taken from breast cancer patients compared with healthy group.

There was a theory about the over- expression in Let-7a level in circulation suggested by many authors (Chin *et al*., 2008; Paranjape *et al*., 2011), the dysfunctional interaction between let-7a with its target mRNA, the KRAS oncogene, was due to a single nucleotide polymorphism in the let-7a complementary site in the KRAS 3'- UTR, prevent let-7a from binding and exerting its tumor suppressor action resulting in over-expression of the oncogene. This failure of binding could lead to lower expression levels of let-7a in tumor tissues, and a reciprocal increase of free let-7a entering the circulation (Johnson *et al*., 2007).

4-3-2-2 Expression of miR-21

In the current study, the expression of miR-21 in plasma of British breast cancer was down expressed compared to healthy women. This down- expression of miR-21in the plasma was not significantly different between breast cancer patients and healthy women and has p= 0.1138, as shown in figure (4-14).

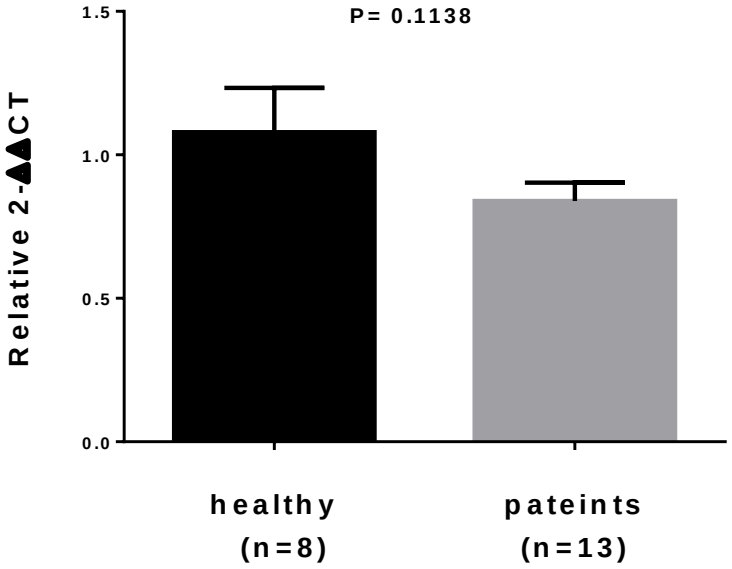


Figure (4-14): Expression of miR-21 in plasma of British women with breast cancer and healthy women volunteers

Dys- regulation of plasma miR-21 in breast cancer patients has not been reported in breast cancer but this dys- regulation of miR-21 reported in ovarian papillary adenocarcinoma cell lines (OVCAR3) (Lou *et al*., 2011).

The study of Kanaan and his partner (2012) found that plasma miR-21 was down- regulated in colorectal cancer patient compared to control and they concluded that this miRNA represent an ideal diagnostic marker for colorectal cancer. They depend on a hypothesis which saying that, when miR-21 level was decreased, significant increase in apoptotic cell death was also observed. Cellular mechanisms that control apoptosis,

cell growth and cell cycle function are efficient fail- safe mechanisms for preventing cancer development. In turn cancer cells only survive if the apoptotic response is disrupted by miR-21, otherwise unrestricted cell proliferation occur (Hanahan and Weinberg, 2000).

Over-expression of miR-21 was mentioned in recent study carried by Kumar *et al*., (2013) in plasma of breast cancer patient they proved that this microRNA (miR-21) was significantly up-regulated in breast cancer patient compared to healthy women.

4-3-2-3 Expression of miR-27a

As presented in figure (4-15), the novel finding in expression of miR-27a was significantly down- regulated in plasma of British breast cancer patient. This down- expression in miR-27a level was detected in breast cancer patient compared with control women with p=0.0004.
All results mean±SD determined in details in appendix II.

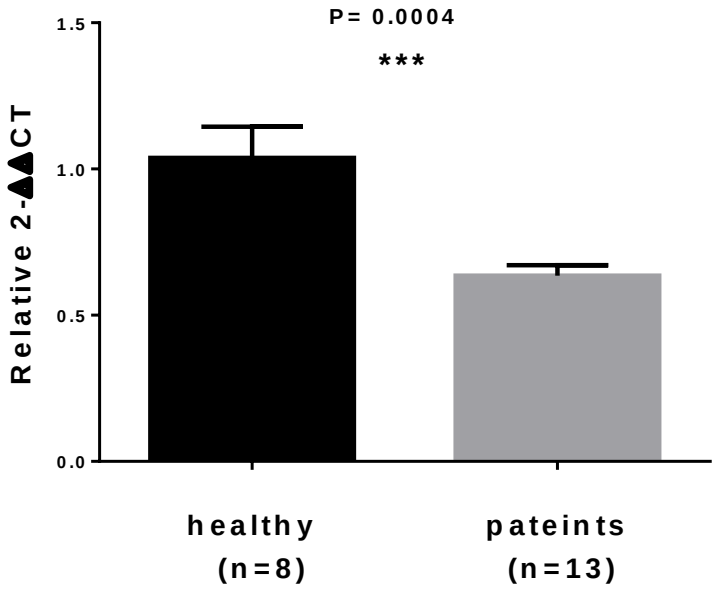


Figure (4-15): Expression of miR-27a in plasma of British women with breast cancer and healthy women volunteers, (***): means significant

MicroRNA-27a reported as breast cancer oncomiR (Liu *et al*., 2009a) that promote cell proliferation in several cancers (Mertens-Talcott *et al*., 2007). MiR-27a expression did not show any significant differences in plasma sample of NSCLC patients compared to healthy group used in the study (Heegaard *et al*., 2012).

In another study they proved that miR-27a expression tends to inhibit the proliferation of gastric cancer cells (Zhao *et al*., 2011). Also the study of Zhou *et al*., (2011) demonstrated that miR-27a was down-regulated in plasma patients with hepatocellular carcinoma compared with healthy volunteers.

4-3-2-4 Expression of miR-222

The expression of miR-222 in plasma of British breast cancer women, significantly down regulation of miR-222 expression level in plasma of breast cancer as indicated in figure (4-16). This down expression in miR-222 in breast cancer was significantly difference compared with healthy women and has p= 0.0286.

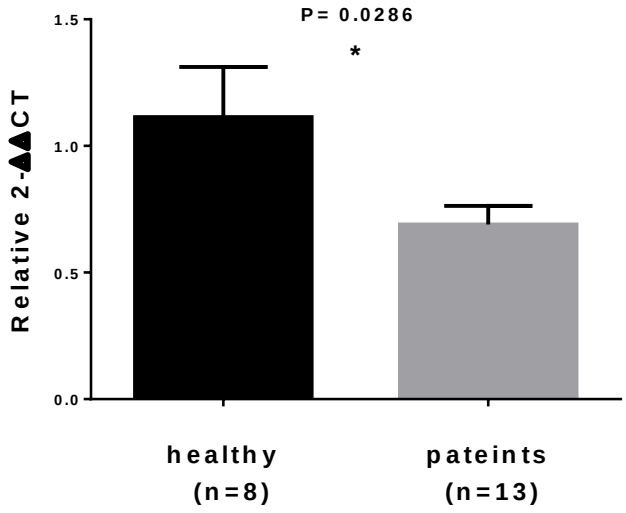


Figure (4-16): Expression of miR-222 in plasma of British women with breast cancer and healthy women volunteers, (*): means significant

MicroRNA-222 represents a good biomarker for breast cancer diagnosis and its role in breast cancer development and progression has been studied (Chen *et al*., 2013)**.** The transition from epithelial to mesenchymal (EMT) phase and breast cancer formation has been contributed to miR-222 role in breast cancer (Di Leva *et al*., 2010; Hu *et al*., 2012).

Another interesting finding about miR-222 up-regulation in breast cancer cell lines theory (Lanbertini *et al*., 2012) the up-regulation of key transcription factor Slug, appears to be crucial since it bind to the miR-221/ miR-222 promoter and it is responsible for the high expression of these miRNAs.

4-3-2-5 Expression of miR-205

Expression of miR-205 was determined in British plasma breast cancer and the result of expression value was shown in figure (4-17). Down- expression in miR-205 in British plasma breast cancer group with significant differences compared with healthy group and has p= 0.1194.

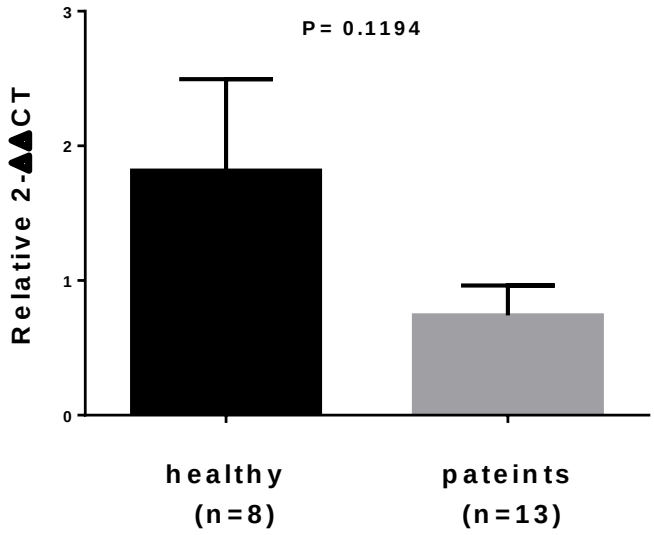


Figure (4-17): Expression of miR-205 in plasma of British women with breast cancer and healthy women volunteers

MicroRNA-205 has dual function as described before as an oncogene or tumor suppressor for example, increased in miR-205 expression has been observed in ovarian cancer (Iorio *et al*., 2007), while the expression of miR-205 was reduced in breast cancer (Wiklund *et al*., 2010).

In ovary cancer, plasma miR-205 was one of the over- expressed miRNAs array were studied and the studies concluded to have a potential biomarker that completes CA-125 in detecting ovary cancer and as a diagnostic and progressive biomarker for cancer (Chen, 2012; Zheng *et al*., 2013).

While in breast cancer cell line, (Paterson *et al*., 2008; Green *et al*., 2010) demonstrated that miR-205 to be included in epithelial to mesenchymal transition (EMT) and in tumor invasion.

4-3-2-6 Expression of miR-218

MicroRNA-218 expression was determined in this study as presented in figure (4-18). The expression of miR-218 was determined in British plasma of breast cancer and healthy group without significant difference in the expression level of miR-218 between healthy individuals and patients, p= 0.2444.

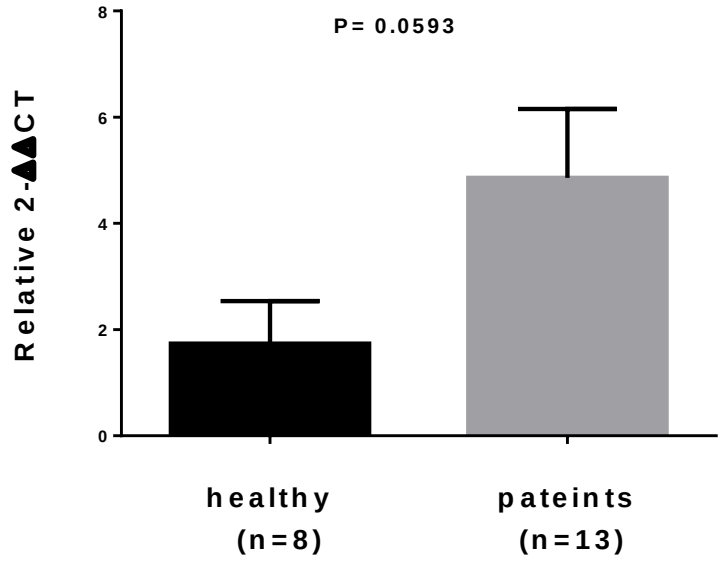


Figure (4-18): Expression of miR-218 in plasma of British women with breast cancer and healthy women volunteers

MicroRNA-218 was mentioned earlier to be encoded by an intron of SLIT2 tumor suppressor (Griffiths- Jones *et al*., 2006), which is known to be associated with the development and progression of several cancers (Calin and Corce, 2006b).

Over-expression in miR-218 was determined in metastatic breast cancer cells that contribute to cell growth and metastasis to bone. This occurs in response to stimulated Wnt signaling and functionally drives Wnt- related transcription and osteoblast differentiation (Hassan *et al*., 2012). While Li *et al*., (2012a), mentioned the down- regulation of miR-218 in plasma of gastric cancer and was evaluating its role in early cancer detection.

Genomic alterations often accounts for down-regulation miRNA expression in malignancies. This was proven by the study of Taterano *et al*., (2011) found that decrease bladder cancer cell lines proliferation, migration, invasion and increase in cell apoptosis were seen in miR-218

transfactant cells suggesting that miR-218 as candidate tumor suppression in human bladder cancer.

4-3-2-7 Expression of miR-378

The novel finding in expression of miR-378 in the British plasma samples was determined in the current study as presented in figure (4-19). The expression of miR-378 in a breast cancer samples was lower than healthy group but statistically it was not significant with p= 0.3530. Un- published study showed expression of miR-378 in cancer patients and this study determines expression of miR-378 in plasma samples of breast cancer. All results mean±SD determined in details in appendix II.

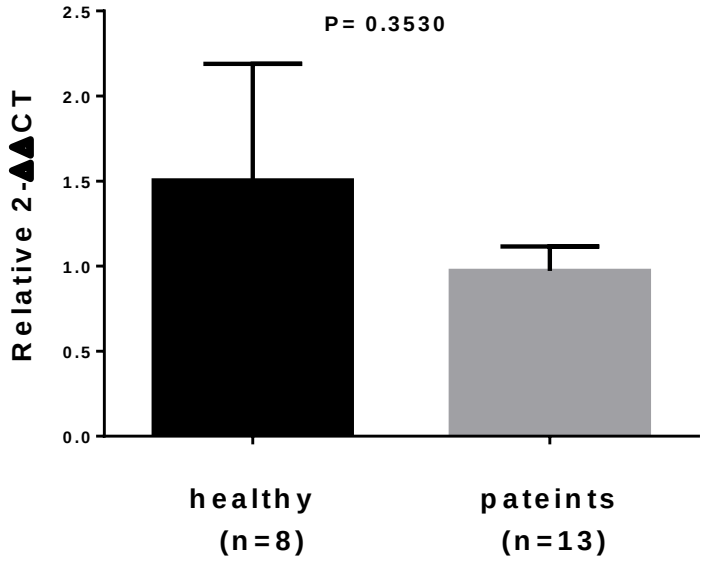


Figure (4-19): Expression of miR-378 in plasma of British women with breast cancer and healthy women volunteers

MicroRNA-378 was shown to be expressed in different cancer cell lines Jiang *et al*., (2005) and Hua *et al*., (2006) suggesting that miR-378 present to be involved in expression of vascular endothelial growth factor (VEGF) by binding to its 3'-UTR region.

Feg and his colleagues (2011) found that miR-378 to be as a novel target of the c-myc oncoprotein that is able to cooperate with activated RAS or Her2 to promote cellular transformation.

MicroRNA-378 act as a molecular switch involved in breast cancer metabolism as described by Eichnert *et al*., (2010) via interference with a well- integrated bioenergetic transcriptional pathway.

4-3-2-8 Expression of miR-15b

MicroRNA-15b expression in plasma of British breast cancer was studied and the result of its expression was presented in figure (4-20). The expression of p miR-15b was shown in plasma samples of breast cancer patients. There was none significant difference in the expression of miR-15b in both cancer and healthy individuals with p=0.5720 between both groups. All results mean±SD determined in details in appendix II.

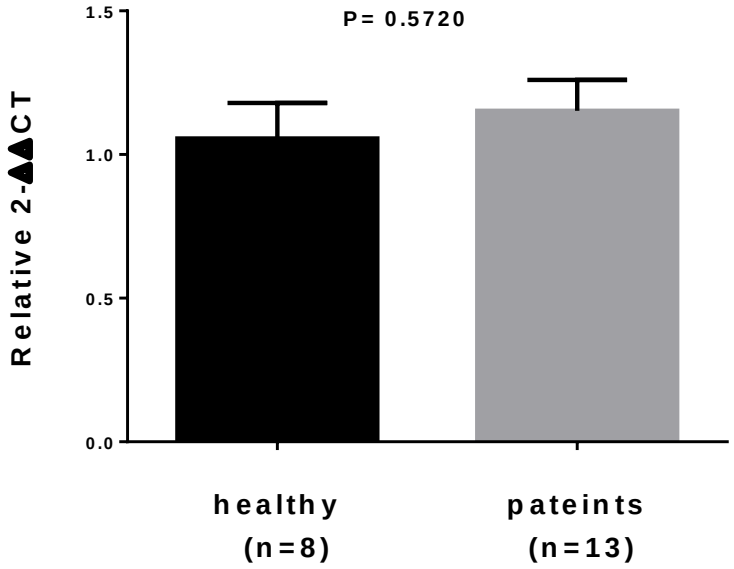


Figure (4-20): Expression of miR-15b in plasma of British women with breast cancer and healthy women volunteers

Plasma expression of miR-15b was measured in colorectal cancer (Kanaan *et al*., 2013); they found that this miRNA can distinguish stage IV colorectal cancer from controls suggesting that miR-15b could provide a better screening for cancer than traditional methods.

Plasma miR-15b was mentioned in two different studies and was given two different results. In lung cancer, (Boeri *et al*., 2011) found that plasma miR-15b was decreased in patients with poor outcome. While in colorectal cancer, (Giraldez *et al.*, 2012) presented that plasma miR-15b was increased in cancer patients compared to healthy group.

4-3-2-9 Expression of miR-26b

A novel over-expression finding of miR-26b in cancer patients was shown in this study as indicated in figure (4-21). The expression of miR-26b in breast cancer patients was significantly different from healthy women plasma with p= 0.0096. All results mean±SD determined in details in appendix II.

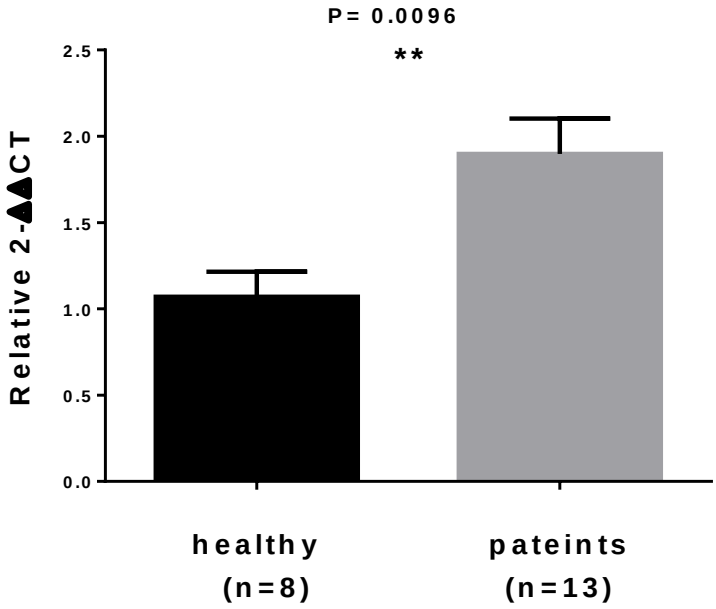


Figure (4-21): Expression of miR-26b in plasma of British women with breast cancer and healthy women volunteers, (**): means significant

MicroRNA-26b was shown to act as tumor suppressor in breast cancer and its over-expression inhibits cellular growth of breast cancer by targeting PTGS gene suggesting its use as potential therapeutic agent for breast cancer (Li *et al*., 2013a). Also miR-26b over-expression shown to induce apoptosis in MCF7 cells by targeting SLC7A11 gene as concluded in the study of (Liu *et al*., 2011).

The expression plasma of miR-26b and miR-21 was higher after tumor removal from head and neck squamous cell carcinoma (HNSCC) patients as observed in the study of Hsu *et al*., (2012), and the study suggested that these miRNAs in pre- and post operative might provide novel tumor marker in circulation of (HNSCC).

TaqMan low- density array screening showed in the study of Soeda *et al*., (2013), nine miRNAs were significantly down-regulated and one of them are miR-26b in the plasma of chronic obstructive pulmonary disease (COPP) patients when compared with normal smokers.

4-3-2-10 Expression of miR-429

The expression of miR-429 was determined in plasma of British breast cancer and the result of the expression was shown in figure (4-22). None significant expression in plasma of British breast cancer compared with healthy plasma was detected in the present study. This represents the first study that determines miR-429 expression level in plasma breast cancer with p= 0.7305.

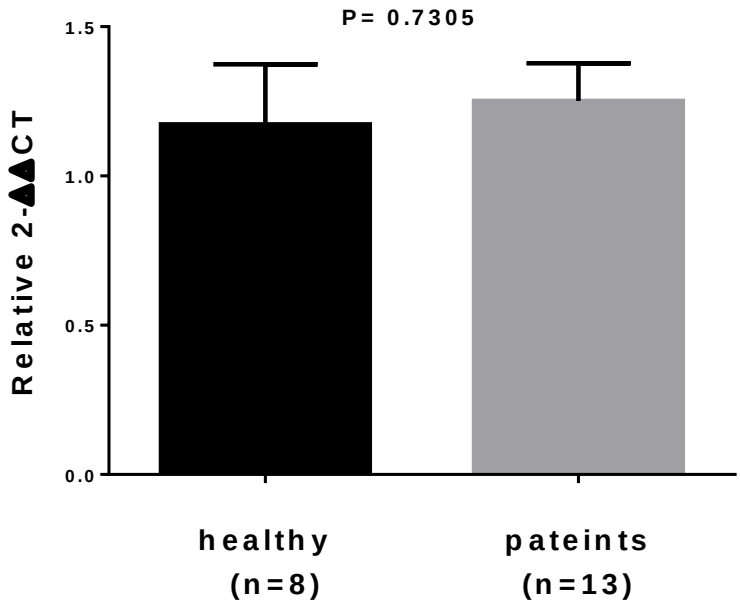


Figure (4-22): Expression of miR-429 in plasma of British women with breast cancer and healthy women volunteers

A study of Attema *et al*., (2013) found that miR-200 gene family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) required for maintenance of the epithelial cell state, but become silenced in mesenchymal cells leading to increased cell motility, proliferation and migration of breast cancer cells.

MicroRNA-429 was shown to be expressed in primary breast cancer and its expression was linked with ER/PR/HER2 expression in the cells (Wu *et al*., 2010).

Over expression of miR-429 in MDA-MB231 breast cancer cells was determined in the study of Nguyen *et al*., (2012) and suggested to be attenuated their invasive satellite morphogenesis in rBM 3- dimensional organotypic culture.

Over expression of miR-429 was shown also in mesenchymal like ovarian cancer cells resulted in reversal of the mesenchymal phenotype MET (mesenchymal- epithelial transition). The study, at the end,

concluded that miR-429 may not only a useful biomarker of EMT in ovarian cancer, but also of potential therapeutic value in ovarian cancer metastasis (Chen *et al*., 2011).

4-3-2-11 Expression of miR-34a

In plasma British breast cancer, the expression of miR-34a was studied and the expression was determined as shown in figure (4-23). Although there was no significant differences and p= 0.508 in plasma miR-34a expression in breast cancer compared with healthy women plasma, the study represent the first study that determine miR-34a expression in plasma of breast cancer. All results mean±SD determined in details in appendix II.

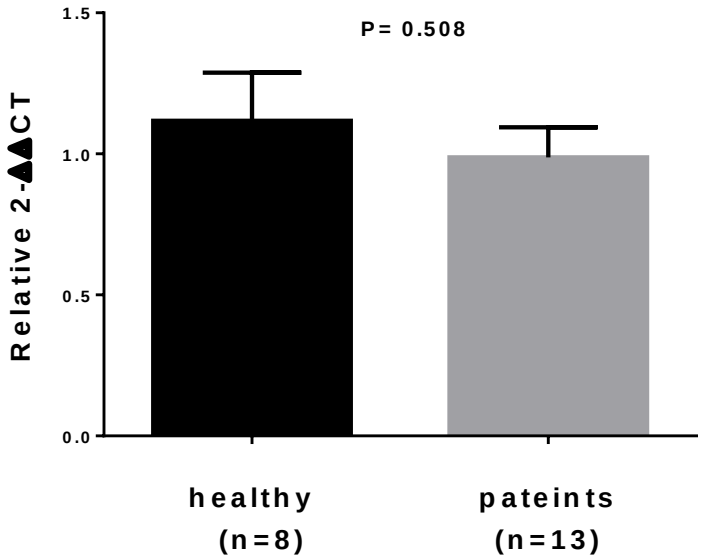


Figure (4-23): Expression of miR-34a in plasma of British women with breast cancer and healthy women volunteers

BCL2 and SIRT1 are the targets of miR-34a; they found to be in reverse correlation with ectopic expression of miR-34a in breast cancer cell lines (Li *et al*., 2013), in this study miR-34a expression was down regulated and function as tumor suppressor.

Kato and his coworker (2009) showed that miR-34a down regulated in breast cancer cell lines which derived from ER/ PR/ Her2 negative (triple negative) tumors, which may reflect the high incidence of *p53* mutation in these subtypes. The same study proved that miR-34 is also required for normal cellular response to DNA damage *in vivo* resulting in altered cellular survival post- irradiation.

Plasma and cerebrospinal fluid (CSF) of miR-34a level was determined in Alzheimer disease (AD) patients. The study Kiko *et al*., (2013) mentioned that a miR-34a level was significantly lower in plasma and CSF of AD patients than in healthy control people. The study concluded that the possibility use of miRNA that detected in plasma and CSF as biomarker for AD.

4-3-2-12 Expression of miR-34b

The expression level of miR-34b was determined in plasma of British breast cancer as shown in figure (4-24). Low expression of miR-34b was detected in plasma of British breast cancer with non significant differences compared with healthy plasma miR-34 expression with p= 0.3324.

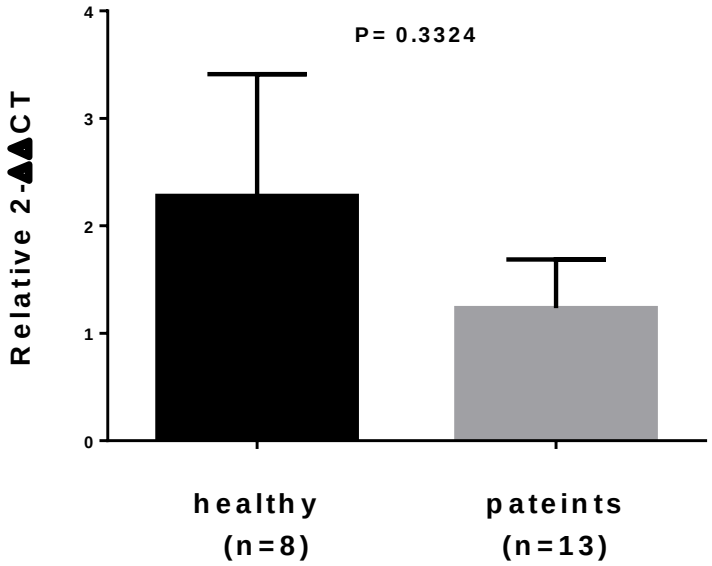


Figure (4-24): Expression of miR-34b in plasma of British women with breast cancer and healthy women volunteers

Expression of miR-34b was shown to be significantly elevated in plasma from Huntington's disease patients and suggesting that this miRNA might be used as biomarkers for Huntington disease (Gaughwin *et al*., 2011). The result also determined that miR-34b is detectable in plasma from small input volumes and is insensitive to freeze- thaw cycle induced RNA degradation.

Another study also determined the plasma level of miR-34b in osteosarcoma patients (Tian *et al*., 2013). Significantly lower miR-34b in plasma osteosarcoma patients compared with healthy subjects was detected and this expression was associated with osteosarcoma risk and related with its metastatic status, suggesting that miR-34b might be a novel biomarker and a potential treatment target for osteosarcoma patients.

In activation of miR-34a and miR-34b/c were concomitant different cancers such as in colorectal, pancreatic, mammary, ovarian,

urothelial and renal cancer cells (Vogt *et al*., 2011). The results showed CpG methylation of miR-34a and miR-34b/c may have diagnostic value.

4-3-3 Expression of microRNA in British breast tissues samples

4-3-3-1 Expression of let-7a

The expression of let-7a in tissues of British women with breast cancer was shown figure (4-25). The down- expression of let-7a was detected in tissues of breast cancer compared with its matched normal tissue borders and did not give any significant differences between both tissues, and p=0.6780. All results mean±SD determined in details in appendix II.

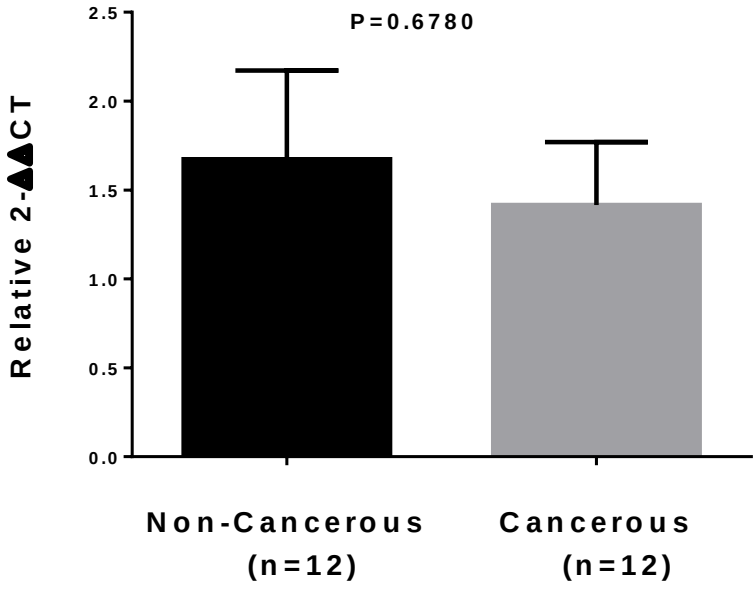


Figure (4-25): Expression of let-7a in tissues of British breast cancer patient and non-cancerous tissues

Studies reported that let-7a was most commonly found to be under-expressed in tumor compared to normal tissue (Boyeinas *et al*., 2010; Zhang *et al*., 2007). Dysfunctional interaction between of let-7a with its target mRNA- the KRAS oncogene, at cellular level, will prevent let-7a from binding to its complementary site in the KRAS 3'-UTR region and

exerting its tumor suppressor effect in over-expression of the oncogene (Chin *et al*., 2008). Any failure of let-7a bind to its oncogene could lead to lower expression level of let-7a in tumor tissues, and a reciprocal increase of free let-7a entering the tumor microenvironment and subsequently the circulation (Johnson *et al*., 2007)

Another role of let-7a has been described that, it play role in promoting tumor growth or proliferation such as reduced let-7a expression in breast cancer was associated with larger tumor size and higher proliferation status (Qian *et al*., 2011), a death in breast cancer patients is primarily due to complications of metastasis a process that let-7a is not correlated with clinically or mechanistically (Foulkes *et al*., 2010).

Cancer cells may evade capillary blood vessels with angiogenic miRNAs; surrounding cells may secrete tumor- suppressive miRNAs, which block tumor growth and propagation (Ma *et al*., 2012). This example can briefly show how miRNA secreted from tissues and circulate in blood and then also evade neighbor tissues.

4-3-3-2 Expression of miR-21

Expression of miR-21 in tissues was determined in current study as shown in figure (4-26). MiR-21 significantly increased in British women with breast cancer tissues compared to normal border tissue. The significant up-regulation of miR-21 was determined in tissues of breast cancer and has a p=0.0014 compared with normal British tissues. All results mean±SD determined in details in appendix II.

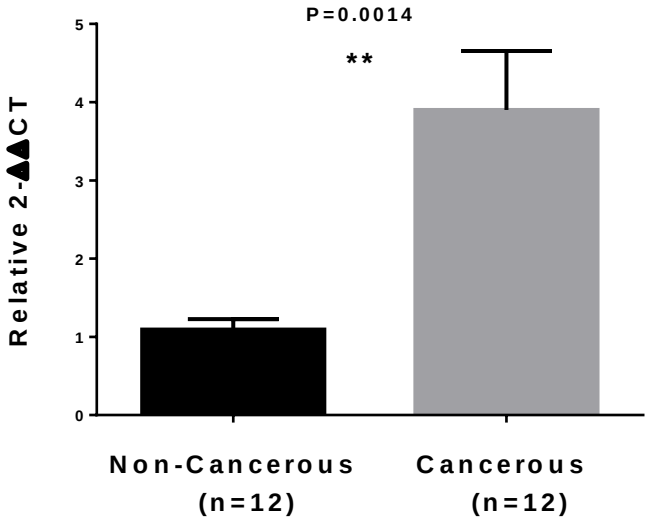


Figure (4-26): Expression of miR-21 in tissues of British breast cancer patient and non-cancerous tissues, (**): means significant

Over-expression of miR-21 was determined in tissues of breast cancer and the possibility of the use of this miRNA to discriminate between breast cancer and non- tumor tissues with high specificity and sensitivity (Rask *et al*., 2011; Mar-Aguilar *et al*., 2013).

Also miR-21 was higher in breast cancer tissues than in normal tissues (Abolins *et al*., 2013); the study showed that different miRNA expression in distinct subtypes of breast cancer patients could be correlated to genetic heterogeneity of breast cancer, different regulatory targets and signaling pathway. They concluded that miR-21 could be a good candidate prognostic marker for breast cancer.

Over-expression of miR-21 has been shown in different studies and this expression could increase cell proliferation, migration, invasion and survival in a variety of cancer cell lines (Mattie *et al*., 2006; Yang *et al*., 2011).

4-3-3-3 Expression of miR-27a

Down regulation in miR-27a expression in breast cancer tissues was represented in figure (4-27). However, none significant differences in miR-27a expression between breast cancer tissues compared to normal border tissues with p=0.1735. All results mean±SD determined in details in appendix II.

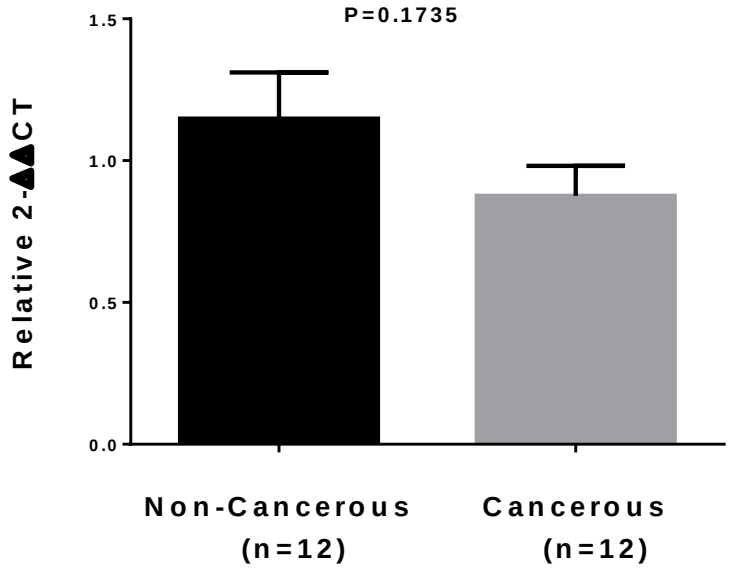


Figure (4-27): Expression of miR-27a in tissues of British breast cancer patient and non-cancerous tissues

Down regulation of miR-27a was shown in the study of Zhao *et al*., (2011) that might inhibit proliferation and drug resistance of gastric cancer cells through regulation of cyclin D1 and P21 and they suggested that miR-27a might be considered as a good target for cancer therapy.

In breast cancer, miR-27a was shown to be involved in the apoptotic response, cell cycle check points and cellular metabolism (Mertens-Talcott *et al*., 2013). High miR-27a expression was shown in the study of Tang *et al*., (2012) to be associated with clinical stages and

poor survival in patients with breast cancer, suggesting miR-27a could be a valuable marker of breast cancer progression.

It has been identified that miR-27a as an oncogenic miRNA and has important role in cancer development for example, it regulates cell growth and division in a dose-dependent manner (Lerner *et al*., 2011). They also have shown to promote human gastric cancer cell metastasis by inducing EMT transition (Zhang *et al*., 2011).

4-3-3-4 Expression of miR-222

The expression of miR-222 in British breast cancer tissue has been detected and the result shown in figure (4-28). The down expression of miR-222 was detected in breast cancer tissues compared to its matched healthy or normal borders breast tissues did not show any significant difference compared to normal breast tissues and gives p= 0.1439.

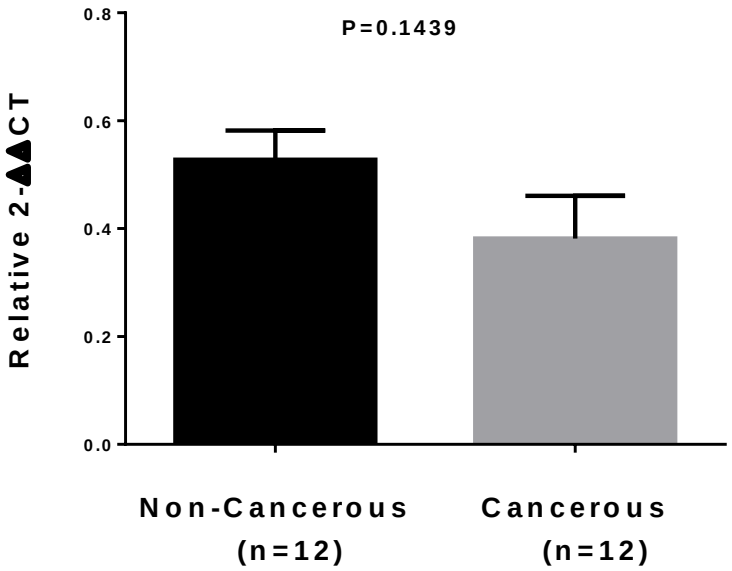


Figure (4-28): Expression of miR-222 in tissues of British breast cancer patient and non-cancerous tissues

MicroRNA-222/miR-221 cluster are increasingly serve as key function in human cells, these miRNAs regulate gene expressions

involved in tumorigenesis, cell proliferation, stress response, angiogenesis, EMT transition and cell differentiation (Chen *et al*., 2010; Guttilla *et al*., 2012).

MicroRNA-222 was over expressed in most cancers which is not what get it in present result. One study mentioned the down regulation of miR-222 in gastrointestinal stromal tumors, (Koelz *et al*., 2011) this study suggested this expression of miR-222 have no role in diagnosis, but it could be considered as a tool for future therapeutic strategies as its role in tamoxifen resistance cells.

Up- regulation of miR-221 and miR-222 has been observed in a number of breast cancer cohorts especially ER-alpha negative primary tumors and in triple negative breast cancer cells (Zhao *et al*., 2008; Radojicic and Zaravinose, 2011).

4-3-3-5 Expression of miR-205

MicroRNA-205 expression was determined in current study and the expression result was shown in figure (4-29). Down expression of miR-205 level was shown in British breast cancer tissues compared to normal tissues. Non-significant differences in miR-205 expression was shown in breast cancer tissues compared to matched normal breast tissues and p= 0.7574.

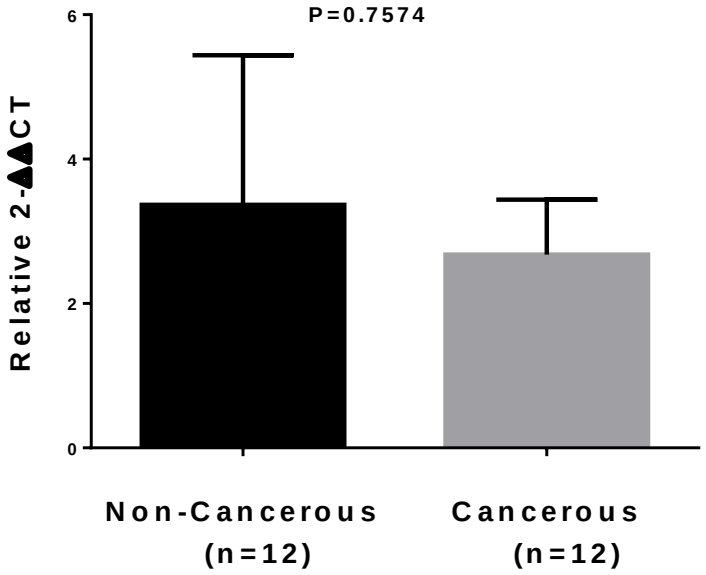


Figure (4-29): Expression of miR-205 in tissues of British breast cancer patient and non-cancerous tissues

MicroRNA-205 was reported to be down regulated in breast cancer tissues in the study of Wu *et al*., (2009), demonstrated that miR-205 could specifically suppress expression of ErbB3 and VEGF-A by directly interacting with putative miR-205 binding site at the 3'-UTR. The result also suggests the role of miR-205 as a tumor suppressor in breast cancer.

Different recent studies mentioned the down regulation of miR-205 in breast cancer tissues. For example, the study Elgamal *et al*., (2013) reported the down regulation of miR-205 expression in breast cancer tissues was associated by targeting HMGB3 (high mobility group box3) gene and suggested that miR-205 represent a good miRNA as a potential therapies for advanced breast cancer.

The deregulation of metastasis- associated miR-205; as described by Markon *et al*., (2013) demonstrated that miR-205 down regulation in primary tumor is associated with clinical outcome in patients with early breast cancer and can differentiate patients with high risk than others.

Other target for miR-205 was determined in the study of Wang *et al*., (2013) which suggested that miR-205 down regulation in breast cancer tissues was found to bind to 3'-UTR of HER3 mRNA and proved that miR-205 play as a tumor suppressor in human breast cancer by posttranscriptional inhibitor of HER3 expression.

4-3-3-6 Expression of miR-218

MiR-218 expression was studied in British breast cancer tissues and the expression was shown in figure (4-30). Down regulation of miR-218 expression has been noticed in breast cancer tissues compared to normal border breast tissues. This down expression in miR-218 in breast cancer was not significantly different from normal border breast tissues and p= 0.1088.

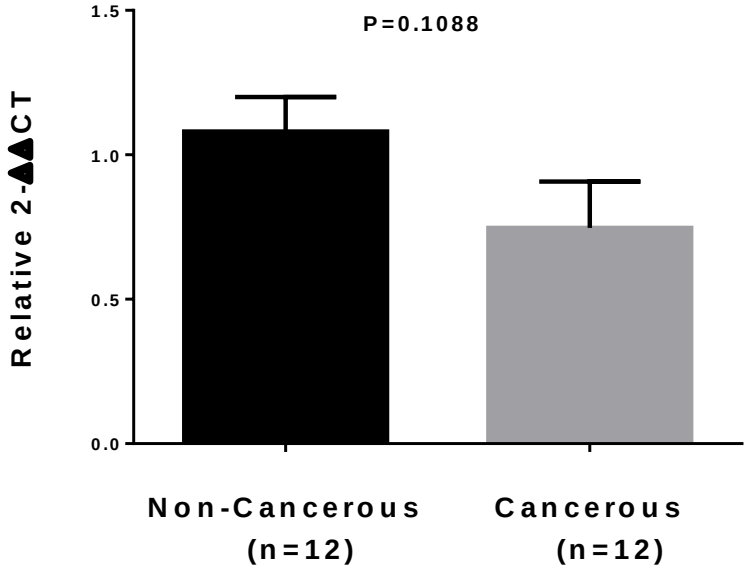


Figure (4-30): Expression of miR-218 in tissues of British breast cancer patient and non-cancerous tissues

MicroRNA-218 was found to be down regulated in the transition stage from ductal carcinoma to invasive ductal carcinoma as determined by the study of Volinia *et al*., (2012). A study carried by using a panel of

miRNAs using deep sequencing method to determine miRNAs in different breast cancer stages.

MicroRNA-218 expression found to be decreased in metastatic breast cancer cells and suppressed migration and invasion through binding Robo1 to its 3' UTR (Yang *et al*., 2012). The study concluded that function of Robo1 regulated by miR-218 may provide a new strategy for inhibition, migration and invasion of breast cancer cells.

4-3-3-7 Expression of miR-378

As presented in figure (4-31) novel finding in the expression of miR-378 in breast cancer tissue was studied in the current thesis. Significant down- expression of miR-378 in British breast cancer tissues was detected compared to healthy normal border breast tissues. This novelty in miR-378 expression in breast cancer tissues has significant differences with normal tissues with p= 0.0006. All results mean±SD determined in details in appendix II.

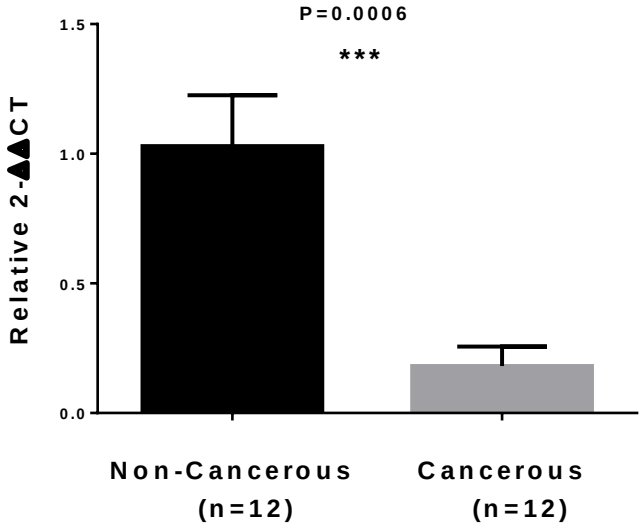


Figure (4-31): Expression of miR-378 in tissues of British breast cancer patient and non-cancerous tissues, (***): means significant

Low expression of miR-378 was detected in the study of Deng *et al*., (2013) in gastric cancer tissues. The low expression in tissues was related to the presence of CpG island methylation on miR-378, this study supports the idea that miR-378 has tumor suppressor properties.

In breast cancer, Lee *et al*., (2007) found that miR-378 enhance cell survival, reduce caspase3 activity and promotes tumor growth and angiogenesis by attenuating the expression of suppressor of fused (Sufu), which is a potential target of miR-378 and Fus-1.

Another study on gastric cancer tissues has been carried out to determine miR-378 expression, the study of Bojian and Haorong, (2012) found that low expression of this miRNA inhibit cell proliferation, cell cycle progression, cell migration as well as invasion also they found that miR-378 act as tumor suppressor in gastric cancer suggesting its novel expression useful in diagnostic and therapeutic role in cancers.

Down regulation of miR-378 has been shown in two different separate studies on colorectal cancer Faltejskova *et al*., (2012) and colonic cancer tissues Wang *et al*., (2010b) both studies suggesting the role of miR-378 as tumor suppressor in cancer.

4-3-3-8 Expression of miR-15b

As displayed in figure (4-32), none significant differences in miR-15b expression in British breast cancer tissues compared with its matched normal border breast tissues, the up-regulation of miR-15b can be seen in the figure in breast cancer tissues and p= 0.3684. All results mean±SD determined in details in appendix II.

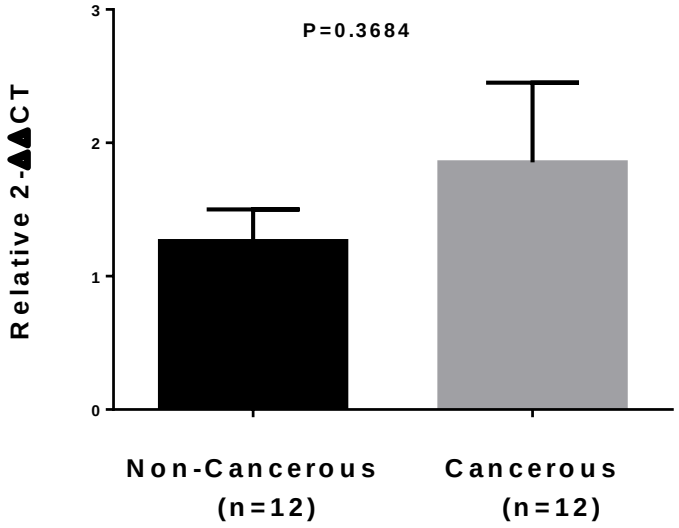


Figure (4-32): Expression of miR-15b in tissues of British breast cancer patient and non-cancerous tissues

Among miRNAs that associated with gain and hypomethylation is miR-15b that shown in the study of Aure *et al*., (2013) miR-15b was up-regulated in malignant breast cancer tissues compared with normal tissues.

In hepatocellular carcinoma Chung *et al*., (2010) found that the over expression of miR-15b shown to reduce Bcl-w expression leading to enhancement of cellular apoptosis and reduction of recurrence risk following curation resection suggesting that miR-15b may be therapeutically useful as an apoptosis sensitivity strategy.

While down regulation in miR-15b has been detected in nasopharyngeal carcinoma as presented in the study of Luqing *et al*., (2011), miR-15b shown to target VEGF and modulate its expression. The study proved that miR-15b has an effect on angiogenic factors in nasopharyngeal carcinoma cells.

4-3-3-9 Expression of miR-26b

As shown in figure (4-33), miR-26b expression in British breast cancer tissue was significantly lower than its matched border normal breast tissues. This expression of miR-26b in breast cancer tissues has significant difference compared with normal tissues and p= 0.0017. All results mean±SD determined in details in appendix II.

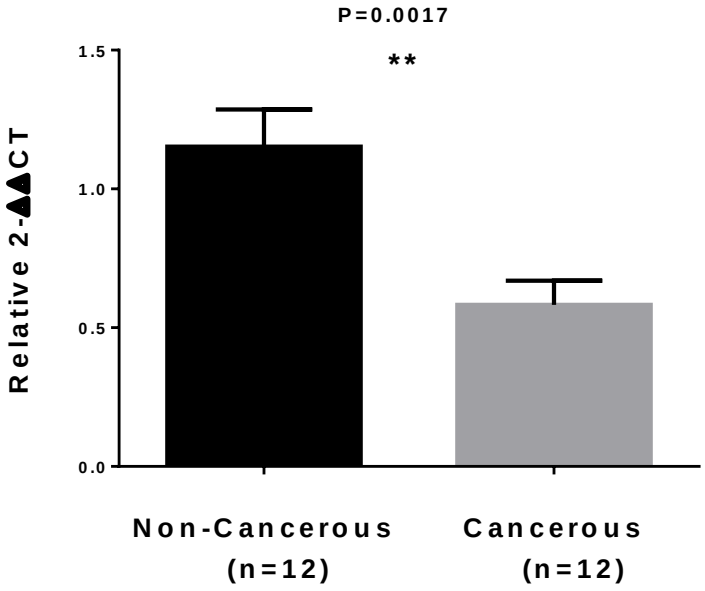


Figure (4-33): Expression of miR-26b in tissues of British breast cancer patient and non-cancerous tissues, (**): means significant

Numerous tumors and normal tissues exhibit different expression of miR-26b during growth, development and tumorigenesis, and miR-26b may participate in various biological processes through imperfect sequence complementary binding between seed region and 3' UTR of target mRNA (Gao and Liu, 2011).

Down regulation of miR-26b has been confirmed in breast cancer and caused fibroblast migration and invasion to increase three folds (Verghese *et al*., 2013). In the study they determined three novel targets for miR-26b (TNK1BP1, CPSF7 and Col12A1) which has shown to be associated with breast cancer recurrence.

Another study carried by Li *et al*., (2013a) also confirmed that miR-26b was down regulated in breast cancer tissue compared with normal tissue and shown to target PTGS2 and inhibit cancer cell proliferation. The study suggested that miR-26b plays a role in tumor suppression and in cancer pathogenesis.

4-3-3-10 Expression of miR-429

In British breast cancer tissues the expression of miR-429 was detected and determined as shown in figure (4-34). The expression of miR-429 was higher in breast cancer tissues compared its matched normal border breast tissues, with significant differences between them and p= 0.0131. All results mean±SD determined in details in appendix II.

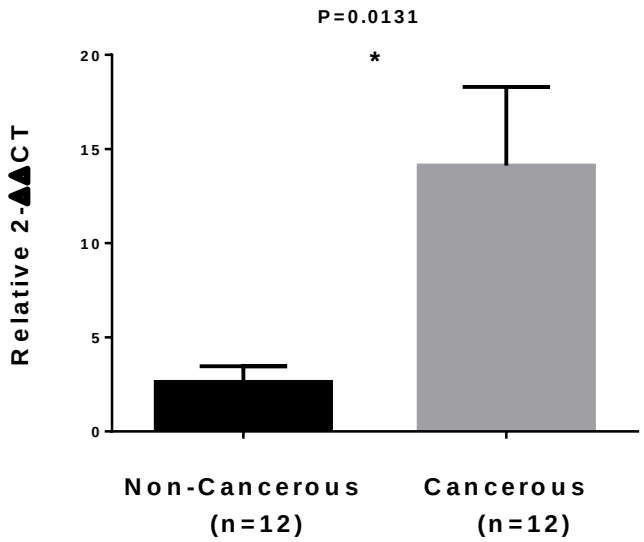


Figure (4-34): Expression of miR-429 in tissues of British breast cancer patient and non-cancerous tissues, (*): means significant

In ovarian cancer, miR-429 expression has been studies and the over expression was detected in mesenchymal- like ovarian cancer cells resulted in reversal of the mesenchymal phenotype (MET) transition. The study concluded the importance of miR-429 as biomarker of EMT in

ovarian cancer and a potential therapeutic value in reducing ovarian cancer metastasis and tumor recurrence (Chen *et al*., 2011).

The dual function of miR-429 was detected in two different studies as an oncogenic in colorectal cancer and as tumor suppressor in gastric cancer, and suggested the differences due to difference of cellular context or alternatively the targeted genes (Huang *et al*., 2013).

MicroRNA-429 expression may modulate the tumorigenesis in gastric cancer and shown to play an oncogenic role (Hashimoto *et al*., 2013). Li and coworker (2013b) found that high expression of miR-429 has been detected in colorectal cancer tissues than in non tumor tissues.

4-3-3-11 Expression of miR-34a

The expression of miR-34a was detected in this study and the expression in British breast cancer and normal tissues was measured as presented in figure (4-35), without any significant differences in the level of miR-34a expression in breast cancer tissues compared with its normal matched tissues with p= 0.4085.

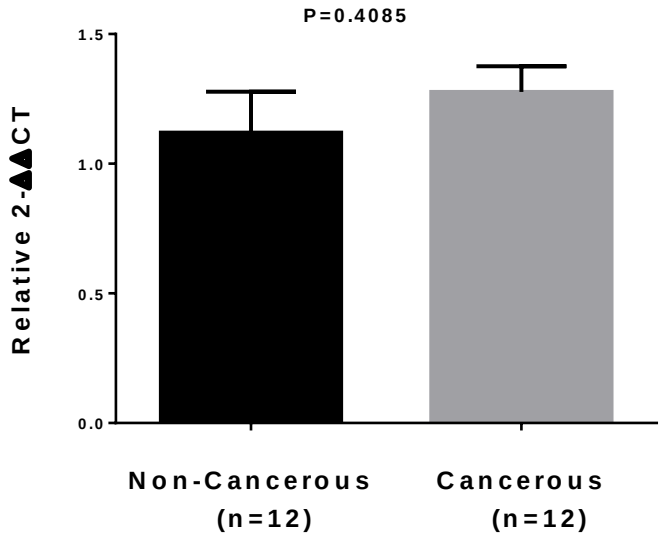


Figure (4-35): Expression of miR-34a in tissues of British breast cancer patient and non-cancerous tissues

Down regulation of miR-34a has been detected in the study of Li *et al*., (2013c) that found 50% of breast cancer tissue samples has down expression in miR-34a compared to matched non malignant breast tissues. This expression suppressed cell proliferation, invasion and induced apoptosis; and shown that miR-34a plays a role as tumor suppressor in breast cancer and the result suggested that miR-34a inhibits proliferation and migration of breast cancer through down regulation of Bcl2 and SIRT2.

In metastatic breast cancer tissues the expression of miR-34a has been determined in the study of Yang *et al*., (2013a) and found to be down regulated and suggested that miR-34a inversely correlated with Fra-1 expression by regulating breast cancer migration and invasion suggesting the therapeutic role of miR-34a in breast cancer.

While in colon cancer, the over expression of miR-34a has been detected in the study of Wu *et al*., (2012) and found to strongly inhibit colon cancer cell migration and invasion which can partially rescued by forced expression of the Fra-1 transcript lacking the 3'-UTR. In this study they mentioned that down regulation of miR-34a was detected as well in 62.5% of colon cancer tissues as compared to matched normal colon tissues and the expression of miR-34a was correlated with DNA- binding activity of *p53*.

4-3-3-12 Expression of miR-34b

The expression of miR-34b has been identified in British breast cancer tissues and the result was presented in figure (4-36). Down regulation of miR-34b has been shown in British breast cancer tissues compared with its matched normal border breast tissues. None significant

differences between breast tissue and normal tissues has been detected with p= 0.4404.

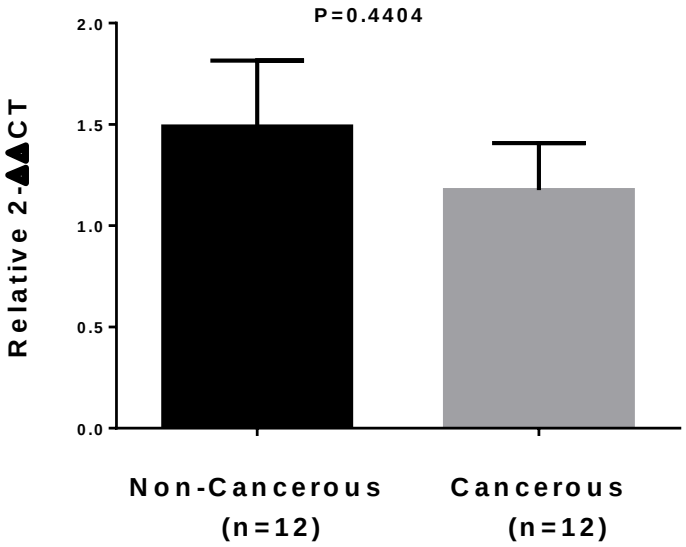


Figure (4-36): Expression of miR-34b in tissues of British breast cancer patient and non-cancerous tissues

Svoboda *et al*., (2012) mentioned that miR-34b expression was negatively correlated with disease free survival and overall survival in triple negative breast cancer patients. The study suggested that miR-34b represent a new prognostic biomarker in triple- negative breast cancer. MiR-34 is a tumor suppressor and found to be silenced by the aberrant DNA methylation of its promoter region in human cancers (Bandres *et al*., 2009), and abnormal expression of this miRNA has been identified in different cancers (Ji *et al*., 2008; Gallardo *et al*., 2009).

Tsai and his colleagues (2011) found that miR-34b tend to be reduced in gastric cancer tissues relative to the adjacent normal tissues. Silencing of miR-34 suggested due to hypermethylated CpG islands during the progression of gastric cancer. This hypermethylation of the miR-34 cluster (b and c) promoter region has been observed in ovarian

cancer and non small lung cancers with their reduced expressions (Corney *et al*., 2010; Gallardo *et al*., 2009).

4-4 Expression of apoptotic genes in British breast cancer tissues

4-4-1 Expression of *p53* mRNA

The expression of *p53* was studied in the current thesis to determine its expression in British breast cancer tissue, and the expression result was determined and presented in figure (4-37). The expression of *p53* in breast cancer tissues was lower than in normal tissues and show significant differences between normal tissue and cancerous tissues and p= 0.0381.

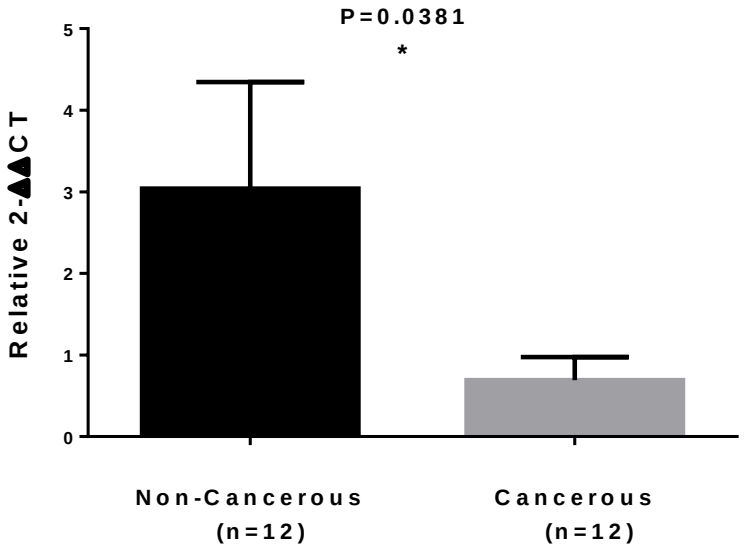


Figure (4-37): Expression of *p53* mRNA in tissues of British breast cancer patient and non-cancerous tissues, (*): means significant

The study of Yang *et al*., (2013b) indicated that *p53* was associated with breast cancer tumor aggressiveness. And the study of Wang *et al*., (2013a) described the inhibition of *p53* by tumor derived factor prostaglandin PGE2 in the breast adipose stroma and suggest that *p53* is decreased in tumor associated stromal cells.

The effects of reduced expression or mutation of *p53* on breast cancer progression were well studied by De Angel *et al*., (2013). The *p53* pathway is generally abnormal in human cancers, either through mutation of the *p53* gene or via modification of *p53* function by interaction with oncogenic cellular or viral proteins (Vousden and Lane, 2007). Somatic *p53* gene mutations found in about 25% of breast cancer and are associated with poor prognosis (Glivier *et al*., 2009), with the highest functional lose <80% in basal like breast cancer tumors (Dumay *et al*., 2013).

Another suggestion about *p53* role in breast cancer, the study of D'Assoro *et al*., (2010) suggested that the loss of *p53* in human breast cancer cells results in EMT- associated stem cell like features.

4-4-2 Expression of *BCL*2 mRNA

As presented in figure (4-38), there was none significance difference in *Bcl*2 expression between breast cancer tissues and adjacent normal tissues and give p= 0.2155. Even without differences in expression of *Bcl2* in both tissues but the expression was higher in breast cancer tissues.

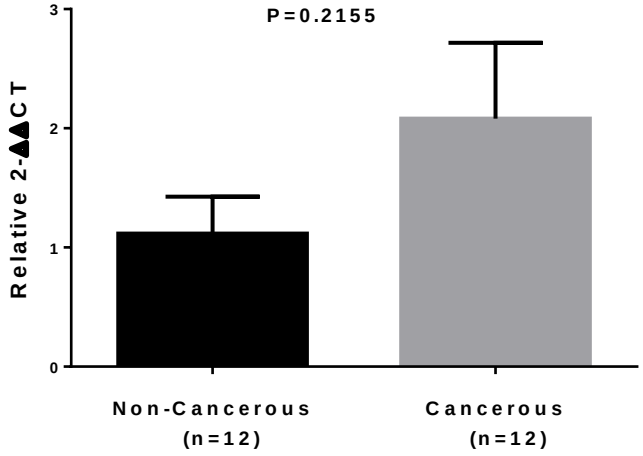


Figure (4-38): Expression of *Bcl2* mRNA in tissues of British breast cancer patient and non-cancerous tissues

The *Bcl*2 provide prognostic marker information in early stage breast cancer in all subgroups (Dawson *et al*., 2010; Hwang *et al*., 2012). The importance of *Bcl*2 expression in breast cancer can differentiate triple negative breast cancer and predict response to chemotherapy (Abdel-Fatah *et al*., 2013).

The study of Korch *et al*., (2013) concluded that up regulation of *Bcl*2 is not involved in lobular breast carcinogenesis and is unlikely to represent an important determinant of tumor development in breast cancer cell lines. Kathan and coworker (2011) reported that *Bcl*2 expression was lower in stroma of precancerous fibro adenoma breast cancer than in those non cancerous tissues

Expression of *Bcl*2 in breast carcinoma tissues significantly found to be lower than in normal tissues (Yu *et al*., 2010a) suggested that *Bcl*2 expression play an important role in the occurrence and development of the breast carcinoma, and any changes in *Bcl*2 expression occurred in the early stage of breast carcinoma.

Expression of *Bcl*2 in breast cancer tissues was significantly correlated with histological grade, nuclear pleomorphism, mitotic counts, estrogen receptor and distant metastasis as determined by Won and coworker (2010).

4-4-3 Expression of *BAX* mRNA

Up regulation in *Bax* gene expression was detected in British breast cancer tissues compared to its adjacent normal tissues as presented in figure (4-39). This expression of *Bax* gene show significant differences between breast cancer tissues with normal healthy border breast tissues and p= 0.0280.

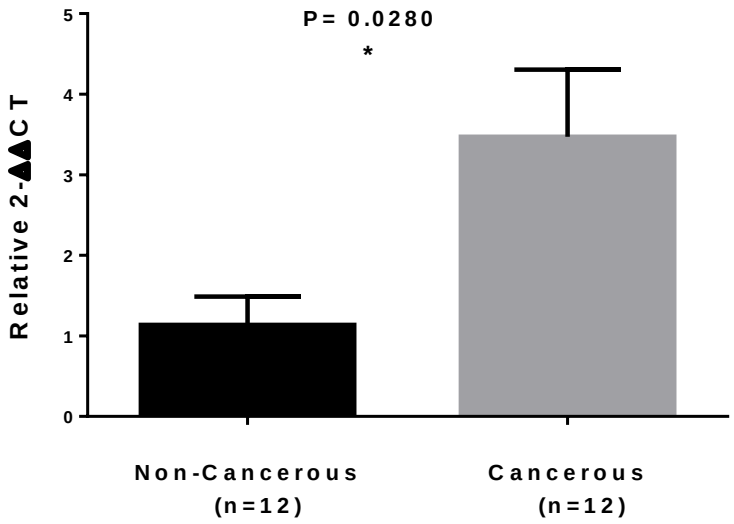


Figure (4-39): Expression of *Bax* mRNA in tissues of British breast cancer patient and non-cancerous tissues, (*): means significant

Up regulation of *Bax* mRNA and apoptotic cell death were more observed in breast cancer depleted of transcriptional factor, CCCTC binding factor as presented in the study of Mendez-Catala *et al*., (2013). They found a novel theory; assuming that increased binding of CTCF to the *Bax* promoter in breast cancer cells in comparison with normal breast cells may be mechanistically linked to the specific apoptotic phenotype in CTCF- depleted breast cancer cells.

In breast cancer tissues, the *Bax* gene expression was over expressed in connection with lower the degree of tumor differentiation suggesting from the study that high *Bax* gene expression provide an early diagnosis and predicts prognosis for breast cancer (Yao *et al*., 2011). Higher expression of *Bax* gene has been detected in study of Jaafar *et al*., (2012) in epithelial breast cancer cells, indicating higher apoptotic activity and subsequent tumor cells apoptosis.

4-4-4 Expression of *BRCA*2 mRNA

The mRNA expression of *BRCA*2 was measured and determined in figure (4-40). Upregulation of *BRCA*2 gene expression has been detected in breast cancer tissues compared with its adjacent normal tissues. Significant differences in *BRCA*2 expression between normal and cancer breast tissues and the p= 0.0424.

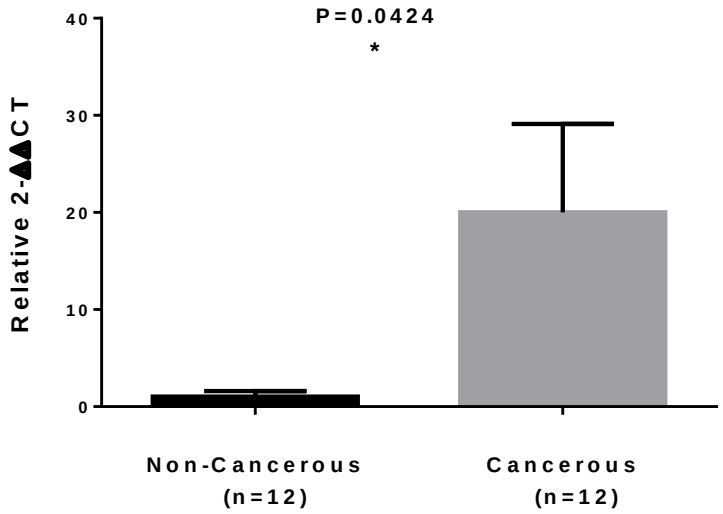


Figure (4-40): Expression of *BRCA2* mRNA in tissues of British breast cancer patient and non-cancerous tissues, (*): means significant

In sporadic breast tumour tissues *BRCA2* expression down expressed in 11% and over expressed in 20%. *BRCA2* over expression correlated significantly with histopathological grade III and was mainly attributed to nuclear polymorphism and mitotic index; the study suggested that the *BRCA2* gene contributes to the proliferation rate in breast tumors (Bieche *et al*., 1999)

The study of Fenale *et al*., (2013) suggested that as *BRCA*2 is an important regulator of homologous recombination process in mammalian cells, so it's down regulation could play critical role in DNA damage repair providing innovative approaches for the development of novel possible therapeutic strategies against breast cancer.

Methylation of *BRCA*2 promoter found to be associated with sporadic breast cancer cells (Cucer *et al*., 2008). Different studies determined the expression of *BRCA*2 in breast cancer, one of these studies was carried by Bellacosa *et al*., (2010) they found that the expression changes in *BRCA*1 was more than *BRCA*2 in cells means that *BRCA*2 is primarily involved in double strand break repair, whereas *BRCA*1 may also bridge double strand repair and signal transduction pathway. Thus from that *BRCA*1 may act as both sensor of DNA damage and as a repair factor while *BRCA*2 found to be involved primarily in actual repair.

4-4-5 Expression of *p21* mRNA

Expression of *p21* gene was determined in British breast cancer and normal tissues and the expression was presented in figure (4-41). Down expression of *p21* show significant differences between cancer and normal breast tissues and has p= 0.0418.

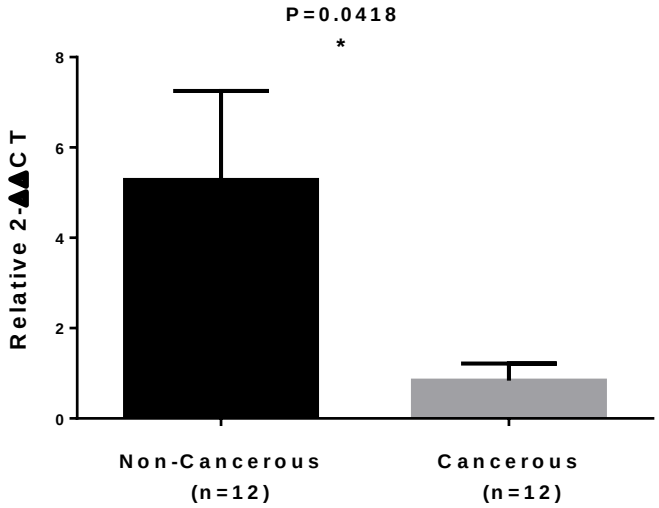


Figure (4-41): Expression of *P21* mRNA in tissues of British breast cancer patient and non-cancerous tissues, (*): means significant

Over expression of *P21* often found in many cancers and their levels of expression was correlated with high tumor grade, poor prognosis and increased metastasis in subsets of carcinoma such as breast, prostate, cervical carcinoma and lymphoma (Abbas and Dutta, 2009).

Dai and his colleagues (2013) found that TGF-beta mediated cyclinD1 and *p21* gene expression in breast cancer cells, which leads to increase breast cancer cell migration and invasion *in vitro*, while blocking of cyclinD1 and p21 cell cycle regulator genes in aggressive human breast tumors shown to reduced in tumor formation and local tumor invasion.

The risk of breast cancer in patients was increased as determined in the study of Askari *et al*., (2013), increased with hypermethylated *p21* promoter suggesting that down regulation of *p21* was due to that promoter hypermethylation.

It has been suggested that *p21* (waf1) was exerts as an anti-apoptotic activity in human breast cancer basal- like cell line HCC 1937 by mediatory of mitochondrial apoptosis (Chen *et al*., 2013a).

4-4-6 Expression of *TWIST* mRNA

Expression of *TWIST* was determined in British breast cancer tissues and its adjacent normal healthy bordered breast tissues. The expression level was presented in figure (4-42); *TWIST* expression level was higher in breast cancer tissues than the level in normal breast tissues. There was significant differences in *TWIST* expression levels between cancer tissues and normal tissues with p= 0.0295.

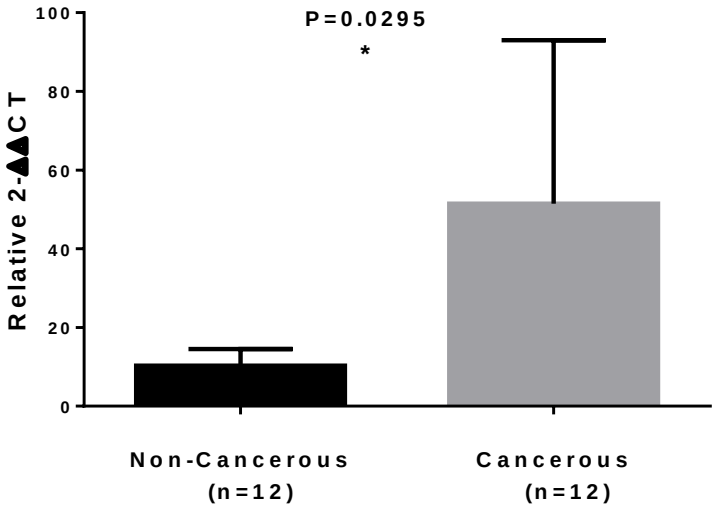


Figure (4-42): Expression of TWIST mRNA in tissues of British breast cancer patient and non-cancerous tissues, (*): means significant

Suppression of *TWIST* expression in highly metastatic mammary carcinoma cells specifically inhibits their ability to metastasize from the mammary gland to the lung (Yang *et al*., 2004).

Over expression of *TWIST* causes chromosomal abnormalities and structural aberration promoting chromosomal instability in MCF7 cell line. Hyper methylation of *TWIST* is a common feature of breast carcinoma (Vesuna *et al*., 2006). It has been found that *TWIST* promoter methylation is more prevalent in cancer cells than normal breast cells and the cells expressing *TWIST* was higher in malignant cells compared to those in healthy tissues (Gort *et al*., 2008).

An increasing or decreasing effect on the *TWIST* expression could be caused by chemotherapy agents in several cell lines could change the cellular fat (Je *et al*., 2013).

Increased expression of *TWIST* was positively correlated with the status of axillary lymph node metastatic and higher tumor- node metastasis stage (Zhao *et al*., 2013). The study then concluded that increased *TWIST* expression was correlated with poor survival and post-

operative relapse-free survival, compared with those for the patients with reduced *TWIST* expression levels. The study suggested that *TWIST* plays a role in the invasion, metastasis and prognosis of breast cancer.

Also *TWIST*1 mRNA expression was determined to be higher in invasive lobular carcinoma (ILC) compared with invasive ductal carcinoma (IDC). *TWIST*1 mRNA expression was not associated with tumor grade and menopausal status (Riaz *et al*., 2012).

Conclusions and Recommendations

Conclusions

1- Estrogen and progesterone hormones levels did not show a perfect indication for early diagnosis of breast cancer and their changes may be due to menopause status, and also CA15-3 biomarker did not represent ideal indicator for early incidence of breast cancer.

2- MicroRNA expression study is very important to be determined in cancer incidence especially in breast cancer.

3- Let-7a and miR-21 were found to be over expressed in serum samples of Iraqi breast cancer women compared with healthy controls. While miR-26b and miR-429 were found to be novel biomarkers in detection of breast cancer in serum samples of Iraqi women because of their down- regulation in patients compared with healthy controls.

4- Let-7a and miR-26b were found to be novel biomarkers in detection of breast cancer in plasma samples of British women because of their high expression in patients compared with healthy controls. Whereas miR-27a and miR-222 were also found to be novel biomarkers in detection of breast cancer in plasma samples of British women because of their down-regulation in patients compared with healthy controls.

5- In tissues of British breast cancer women, the expression of miR-21 and miR-429 (the novel) were up-regulated while miR-378 and miR-26b (the novel) were found to be down-regulated, they were also regarded as novel biomarkers in detection of breast cancer in tissues.

6- The expression of apoptotic genes in British breast cancer women *P53*, *P21* and *TWIST* were significantly down regulated in breast cancer tissues while *Bax* and *BRCA2* were significantly up-regulated in the same groups.

Recommendations

1- Further studies are required to identify the expression of these microRNAs (used in current study) in serum of other types of cancers for example lung cancer, prostate cancer, gastric cancer and others, as an early diagnosis of cancer using microRNAs

2- Investigate the expression of other novel microRNAs in plasma/ serum of breast cancer women as early diagnosis in detection of breast cancer

3- Compare the expression of microRNAs in breast cancer and other types of cancer in different province of Iraq

4- Study the correlation between obesity with miRNA expression in a way to determine miRNA expression changes in obese people.

5- Determine the correlation between age and telomere expression with miRNA expression to determine the role of miRNA in telomere shortening in cancer cells.

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الخلاصــة

تهدف هذه الدراسة إلى تحديد التعبير عن جزيئات microRNAs والمؤشرات الحيوية للتشخيص المبكر للكشف عن سرطان الثدي. لتحقيق هذا الهدف، تم جمع ما مجموعه خمسة عشر عينة مصل من النساء العراقيات المصابات بسرطان الثدي و ستة من عينات المصل تم جمعها من نساء متطوعات سليمات كمجموعة سيطرة. في هذه عينات المصل العراقية تم تحديد مستويات هرمون الاستروجين و البروجسترون و CA15 -3 لدراسة العلاقة بين هذه المعايير و حالات الإصابة بسرطان الثدي في مصل النساء العراقيات. وأظهرت النتائج أن هناك علاقة سلبية ($r^2=^-0.4$) بين مستوى هرمون الاستروجين وعمر المريضات المصابات بسرطان الثدي، في حين ليست هناك علاقة ذات دلالة إحصائية بين مستوى هرمون البروجسترون وعمر المريضات المصابات بسرطان الثدي، و كذلك عدم وجود ارتباط كبير بين مستوى CA(15-3) وعمر المريضات مقارنة مع السليمات مما يعني أن مستوى هرمون الاستروجين قد انخفضت مع عمر المريضات.

من ناحية أخرى، تم تحديد التعبير عن العديد من microRNAs في عينات مصل النساء المصابات بسرطان الثدي والسليمات باستخدام تقنية qPCR. هذه microRNAs المستخدمة كانت تتضمن (let-7a، miR-21، miR-222، miR-205، miR-218، miR-378، miR-15b، miR-26b، miR-27a، miR-429، miR-34a، miR-34b و miR-191 (كسيطرة)). وأظهرت النتائج أن هناك زيادة معنوية ($P <0.05$) في التعبير عن let-7a و miR-21 في مصل مريضات سرطان الثدي مقارنة مع السليمات حيث كان مقدار التغير (٢.١ و ٣.٥) على التوالي، بينما هناك انخفاض معنوي ملحوظ ($P <0.05$) في التعبير عن miR-26b و miR-429 في مريضات سرطان الثدي مقارنة مع الاصحاء حيث كان مقدار التغير (٠.٥) لكلا الـ miRNAs والتي تعد من المؤثرات الجديدة في الكشف عن سرطان الثدي، اما باقي الـ miRNAs فلم تظهر أي اختلاف كبير في التعبير بين مريضات سرطان الثدي والنساء السليمات.

تمت دراسة تعبير للـ miRNAs أيضا في ثلاثة عشر عينة من البلازما لنساء بريطانيات مصابات بسرطان الثدي وثمانية متطوعات اصحاء. وأظهرت النتائج أن هناك

زيادة معنوية (P <0.05) في التعبير عن let-7a و miR-26b في عينات البلازما للنساء المصابات بسرطان الثدي مقارنة مع بلازما النساء السليمات حيث كان مقدار التغير (١.٥ و ٢.٠) على التوالي، في حين أن تعبير miR-27a و miR-222 كان منخفض معنويا (P <0.05) حيث كان مقدار التغير (٠.٦) لكلاهما.

من ناحية أخرى استخدامت ثلاثة عشر عينة من أنسجة سرطان الثدي البريطانية وأنسجتها المتطابقة لمجموعة سيطرة لتحديد التعبير للـ miRNAs. وكشفت النتائج أن miR-21 و miR-429 (الجديدة) ازدادت معنويا (P <0.05) في التعبير في أنسجة سرطان الثدي مقارنة مع الأنسجة الطبيعية وكان مقدار التغير (٤ و ١٤) على التوالي، ولكن التعبير كان منخفضا معنويا (P <0.05) لـ miR-378 و miR-26b (الجديدة) في انسجة سرطان الثدي وكان مقدار التغير (٠.٢ و ٠.٦) على التوالي مقارنة مع أنسجة الثدي الطبيعية المتطابقة.

كان الجزء الأخير من هذه الدراسة لتحديد التعبير عن الجينات apoptotic في سرطان الثدي البريطانية عينات الأنسجة المتطابقة وأنسجه الثدي الطبيعية. هذه الجينات apoptotic المستخدمة في هذه الدراسة كانت (*P53*، *P21*، *Bax*، *Bcl2*، *BRCA2* و TWIST). وأشارت النتائج إلى أن تعبير *P53*، *P21* و*TWIST* كان انخفاضها معنويا (P <0.05) في أنسجة سرطان الثدي مقارنة مع أنسجة الثدي الطبيعية مع مقدار التغير (٠.٩، ١.٠ و ١.٠) على التوالي، في حين كان هناك ازدياد معنوي(P <0.05) في تعبير عن *Bax* و *BRCA2* حيث كان مقدار التغيير (٣.٥ و ٢٠.٠) على التوالي.

في الختام، أشارت النتائج إلى أن هناك إمكانية للكشف عن التغيرات في مستوى تعبير الـ microRNAs كمؤشرات حيوية مفيدة للكشف والتشخيص المبكر لسرطان الثدي.