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Genetic and Biochemical Study in a sample of Iraqi women with breast cancer above 40 Years

A Thesis

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By

Sara Saleh Hassan Sadkan

B. Sc., Biotechnology, College of Science, 2006
Al-Nahrain University

Supervised

Prof. Dr. Amina N. Al-Thawiny *Prof. Dr. Waleed H. Yousif*

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Supervisors Certification

We certify that this thesis was prepared under our supervision at the Department of Biotechnology, College of Science, Al-Nahrain University as partial requirements for the Degree of Science in Biotechnology.

Signature:
Supervisor:Dr. Amina N. AL-Thaweni
Degree: Professor
Date:

Signature:
Supervisor:Dr. Waleed H.Yousif
Degree: Professor
Date:

In review of available recommendations, I forward this thesis for debate by Examining Committee.

Signature:
Prof. Dr. Kadhim M. Ibrahim.
Head of Biotechnology Department
College of Science
Al-Nahrain University

Committee Certification

We, the examining committee, certify that we have read this thesis and examined the student in its contents and that according to our opinion is accepted as a thesis for the degree of Master of Science in Biotechnology.

Signature:
Name:
Scientific Degree:
Date:
(Chairman)

Signature:
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Signature:
Name:
Scientific Degree:
Date:
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Signature:
Name:
Scientific Degree:
Date:
(Supervisor)

Signature:
Name:
Scientific Degree:
Date:
(Supervisor)

I herby certify upon the decision of the examining committee

Signature:

Name: Laith Abdul-Aziz Al-Ani

Scientific Degree: Assistant Professor

Title: Dean of College of Science

بسمِ ٱللَّهِ ٱلرَّحمَنِ ٱلرَحِيمِ

۞ إِنَّا عَرَضْنَا الْأَمَانَةَ عَلَى السَّمَاوَاتِ
وَالْأَرْضِ وَالْجِبَالِ فَأَبَيْنَ أَن يَحْمِلْنَهَا
وَأَشْفَقْنَ مِنْهَا وَحَمَلَهَا الْإِنسَانُ إِنَّهُ كَانَ
ظَلُوماً جَهُولاً ۞

صَدَقَ اللهُ الْعَظِيمُ

سورة الأحزاب الآية 72

الأهداء

- الى خيمتي الشماء، الى القلب الحنون ،الى مَنْ كانَ شمعةً تنير دربي محبةً وحنان وعيناً سَهرَتْ لأغفو بأمان ، الى ارضٍ ورمزٍ وحضارة ، يامن علمتني أن أقف بعزةٍ وثبوت في كل حين... **اليك والدي الحنون**.
- الى املٍ أنار دنياي منذ ولدت وستبقى حياً فيَ للمماتِ ، الى من علمتني السجود لله وأنْ أبكي بحضرته ليعينني على مصاعب الدُنيا والآن أسجُدُ لطلب الرحمةً لها، الى من سقتني من روحها قبل لَبنها، الى يدٍ سندتني بأول خطوة ، أذنٌ نغمت لأول كلمة وعين زغردت لاول طلة الى صدر لم اجد عنه البديل ، الى من صرت ذاتها بعد أن إمتلكت مني الدمع والذات ، وكيف عسايَ أن أشكر مَنْ غفى في روحي ورأه الناس حياً في عيني.. **إليك أمي حبيبتي عساك أن تكوني في جنات النعيم إن شاالله**.
- الى من سرنا سويةً ونحن متشابكي الأيدي ، الى سندي وقوتي وعزمي في الدنيا، الى زهورٍ أوردت لتزهو في عيني يوم تلو يوم ... إليكن أخواتي **(نورا ، مروه ،منار ، ريام)**.
- الى من عرفت معها معنى الصداقه ،الى التي لم ولن استغني عن صداقتها مادمت حيه ،الى رفيقت دربي وزهره حياتي.... **همسه**
- الى أول من علمني حرفا وحتى آخر من تلقيت على يده علما.

أهدي ثمرة جهدي المتواضع

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Sarah Salih Hassan

Summary

Breast cancer is the commonest cancer affecting women worldwide. Different studies have dealt with the etiological factors of that cancer aiming to find a way for early diagnosis and satisfactory therapy. The present study investigated the relationship between genetic polymorphisms of BRCA1 & BRCA2 genes and evaluation of some etiological risk factors among breast cancer patients in Iraq. This investigation was carried out on 25 patients (all were females) who were confirmatory for breast cancer by histopathological examinations attended from AL-Kadhemya Teaching Hospital in Baghdad and 10 of apparently healthy women were used as a control. All women (patients and control) their age above 40 years. Ages, fimaily history, blood group, age at menarche and hormonal factor (estrogen) were taken into account as risk factors.

Blood samples were collected from 10 breast cancer women and 10 control women for determination lipid profile and estrogen level. Results have showed a significant increase in estrogen levels in (70%) of patients which may reflect a positive association between estrogen and breast cancer, while lipid profiles {Triglyceride (TG), Total cholesterol (TC) and High density lipid (HDL)} showed no significant association with breast cancer.

The Wizard Promega kit was used for DNA isolation from fresh biopsy of women with breast cancer patients and normal individuals. By this method a suitable quantities of DNA approximately (50 µl) were obtained from 100 µg -200 µg of fresh biopsy taken from women breast. The purity of isolated DNA ranged from (1.7–1.9). The extracted DNA was successfully used in amplification of BRCA1 & BRCA2 genes by PCR

and some mutation were detected. The outcome of genetic analysis indicated that the percentage of 185delAG mutation was 16 (4 patients) whereas, the percentage of 5382insC mutation was 32 (8patients) in BRCA1 gene and the third mutation 6174delT in BRCA2 present in 3 patients only (12%). The study demonstrated that the frequency of BRCA1 mutation was (48%) higher than BRCA2 (12%) in this sample of Iraqi women with breast cancer.

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Lists of Aberrations

CYP2D6……	Cytochrom P450 genes 6
DNTPs……	Deoxy nucleotide triphosphates
DNA……..	Deoxyribose Nucleic Acid.....
D.W……...	Distilled water
DCIS……	Ductal carcinoma *in situ*
Erb-2……	Erythroblastic leukemia viral oncogene homologue 2
EDTA……	Estrogen receptor and *ER* gene
ER……	Estrogen receptor and *ER* gene
FISH……	Fluorescent in-situ hybridization
BRCA1 &BRCA2……	Genes (Breast cancer gene 1 and 2)
EGF-R……..	growth factor receptor
HDL……..	High density lipid
HRT……..	hormone replacement therapy
HER2……	human epidermal growth factor receptor 2
IHC……	Immenohistochemistry
185-KDa……	Kilo Dalton
LCIN……	Lobular carcinoma *in situ*
LDL……..	Low density lipid
C-MYC……	Myelocytomatosis viral oncogene homologue
O.D……	optical density
PTEN……	Phosphates Tensin homolog
P53……	Protein 53 kilo Dalton
RIBC5 ……..	Raculoviral IAP reapt congaing 5
SHBG……	sex hormone binding globulin
SSCP……	Single strand conformation polymorphism

PCR……..	The polymerase chain reaction
TC…….	Total cholesterol
TG…….	Total triglycerides
TNM…….	Tumor, Nodal, and Metastasis Staging
WHI……..	Women’s Health Initiative

Chapter One

Introduction

And

Literature Review

1.1.Introduction

Cancer occurs as a result of mutations, or abnormal changes, in the genes responsible for regulating the growth of cells and keeping them healthy. These mutations can “turn on” certain genes and “turn off” others in a cell. That changed cell gains the ability to keep dividing without control or order, producing more cells just like it and forming a tumor (Marisa, 2008). Malignant tumors are cancerous because malignant cells eventually can spread beyond the original tumor to other parts of the body (Brian *et al*., 2008).

Breast cancer is a common cancer among women in the world and the single leading cause of death in women aged 40 to 49. It is estimated that by the year more 2 million women die in the world from breast cancer (Black *et al*., 2007). In the United States about 178,480 women affecting by breast cancer (American Cancer Society 2007). Also, in the United Kingdom, where the age standardized incidence and mortality is the highest in the world, the incidence among women aged 50 approaches two per 1000 women per year, and the disease is the single commonest cause of death among women aged 40-50, accounting for about a fifth of all deaths in this age group (Landis *et al*., 1998).

In Iraq, where the population was exposed to high levels of depleted uranium following the first and second Gulf Wars, breast cancer is the most common cancer type in females. Over the last ten years, there has been a three-fold increase in the incidence of breast cancer (Al-Azzawi, 2006). Breast cancer alone is accounted for (31%) of all new cancer cases among females in Iraq (Al Hasnawi, 2008). No really attempt was done to study this problem on the molecular basis while some studies concerning breast cancer

were accomplished using cytogenetic techniques & Random polymorphic DNA amplification (Jaffer, 1999; Jasim, 2004).

A breast cancer gene is a gene in which germ line mutations or polymorphisms confer increased susceptibility to breast cancer (Polyak, 2002). Two breast cancer susceptibility genes are BRCA1 and BRCA2. The presence of a mutation in either BRCA1 or BRCA2 will increase an individual's lifetime risk of developing breast cancer to 60-85% (Rebbeck *et al*., 2001).

Estrogen, it is essential for the normal growth and development of the breast and tissues important for reproduction. It is important for childbearing and helps regulate a woman's menstrual cycles. Higher exposure to estrogen is implicated in the formation breast cancer. Estrogen can induce caners to form in two ways, as a genotoxin and a mitogen. A genotoxin or mutagen directly damages the DNA (causes mutations), initiating a process that leads to the formation of cancer cells. A mitogen causes cells to proliferate, that is, to multiply through division (mitosis). Each a time a cell divides to form two cells, it must replicate its DNA. The stimulation of proliferation (mitogenesis) that estrogen causes increases the chances those abnormal cells will grow into malignant tumors (Miller, 2003).

Also the changes in lipid profile have long been associated with cancer because lipids play a key role in maintenance of cell integrity. Malignant proliferation of breast tissue in women has been associated with changes in plasma lipid and lipoproteins levels (Lane *et al*., 1995). It has been postulated that changes in the concentration of serum lipidsin the breast cancer patients could result in increase production of tumor necrosis factor (Knapp *et al*., 1991).

Up to our knowledge, no studies in Iraq have dealt with BRCA1 & BRCA2 mutation and there relation with breast carcinogenesis, further, this work was concerned with hormonal status in breast cancer patients.

The current study aimed to:-

- Investigate the presence of (185del AG,5382ins C and 6174delT) in BRCA1 & BRCA2 genes in Iraqi women above 40 years with breast cancer.
- To evaluate the relationship of estrogen hormone level & lipid profile in women with breast cancer.

Literatures Review

1.2. Background

Cancers are caused by a series of mutations. Each mutation alters the behavior of the cell somewhat. Cancer is fundamentally a disease of regulation of tissue growth. In order for a normal cell to transform into a cancer cell, genes which regulate cell growth and differentiation must be altered (Croce, 2008). Genetic changes can occur at many levels, from gain or loss of entire chromosomes to a mutation affecting a single DNA nucleotide. Nearly all cancers are caused by abnormalities in the genetic material of the transformed cells. These abnormalities may be due to the effects of carcinogens, such as tobacco smoke, radiation, chemicals, or infectious agents. Other cancer-promoting genetic abnormalities may be randomly acquired through errors in DNA replication, or are inherited, and thus present in all cells from birth. The heritability of cancers is usually affected by complex interactions between carcinogens and the host's genome (Kinzler, 2002). There are two broad categories of genes which are affected by these changes.

1- Oncogenes are mutated forms of genes that cause normal cells to grow out of control and become cancer cells. They are mutations of certain normal genes of the cell called proto-oncogenes. Proto-oncogenes are the genes that normally control how often a cell divides and the degree to which it differentiates (or specializes). When a proto-oncogene mutates (changes) into an oncogene, it becomes permanently "turned on" or activated when it is not supposed to be. When this occurs, the cell divides too quickly; this can lead to cancer (Pierotti *et al*., 2004). The chance of cancer cannot be reduced by removing proto-oncogenes from the genome, even if this were

possible, as they are critical for growth, repair and homeostasis of the organism. It is only when they become mutated that the signals for growth become excessive (Chang *et al*., 2006).

2- Tumor suppressor genes are normal genes that slow down cell division, repair DNA mistakes, and tell cells when to die (a process known as apoptosis or programmed cell death). When tumor suppressor genes don't work properly, cells can grow out of control, which can lead to cancer. About 30 tumor suppressor genes have been identified, including p53, BRCA1and BRCA2 (Park, 2003). An important difference between oncogenes and tumor suppressor genes is that oncogenes result from the activation (turning on) of proto-oncogenes, but tumor suppressor genes cause cancer when they are inactivated (turned off). Another major difference is that the majority of oncogenes develop from mutations in normal genes (proto-oncogenes) during the life of the individual (acquired mutations) while abnormalities of tumor suppressor genes can be inherited as well as acquired (Matoba *et al*., 2006).

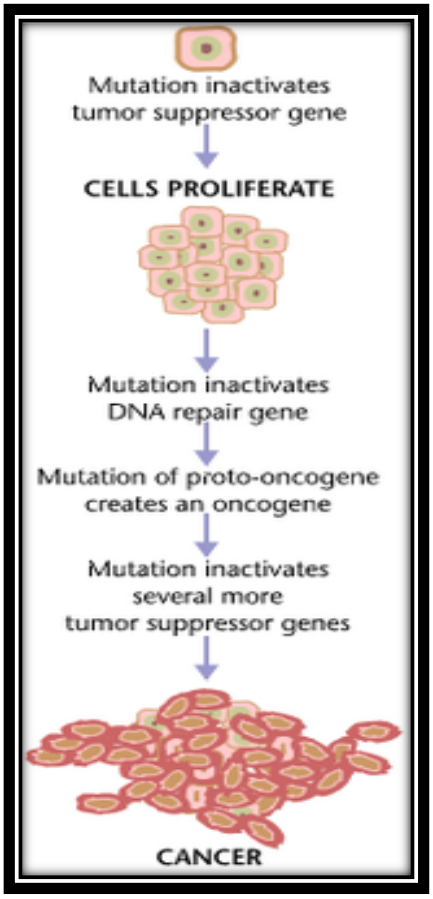

Figure (1-1) Cancers are caused by a series of mutations. Each mutation alters the behavior of the cell somewhat.

1.3. Breast cancer anatomy

Breast cancer is the malignant tumor caused by uncontrolled growth of cells in the breast. It is the most common cancer among women (Madhavan *et al*., 2002). Most breast cancers begin in the milk ducts. These ducts connect the milk-producing glands (called lobules) to the nipple. Some breast cancer begins in the lobules themselves, and the rest begins in other tissues. The diagram shows where these body parts are within the breast fig (1 -1). Once the cells have broken through the wall of a duct or lobule, the cancer is called invasive. If the cells then travel into the lymphatic vessels of

the breast, they may be carried to other parts of the body so that the cancer spreads. This process is called metastasis. Not all breast tumor are considered to be cancer because certain type of large cell collection cannot spread or threaten a persons life, this type of tumor is called a benign tumor (Susan and Rumzi, 1999) while the cancerous cells remain inside the duct or lobule, these cancers are called *in situ*. After a diagnosis of *in situ* cancer, there is currently a 5-year survival rate of 97% (AmericanCancer Society, 2006).

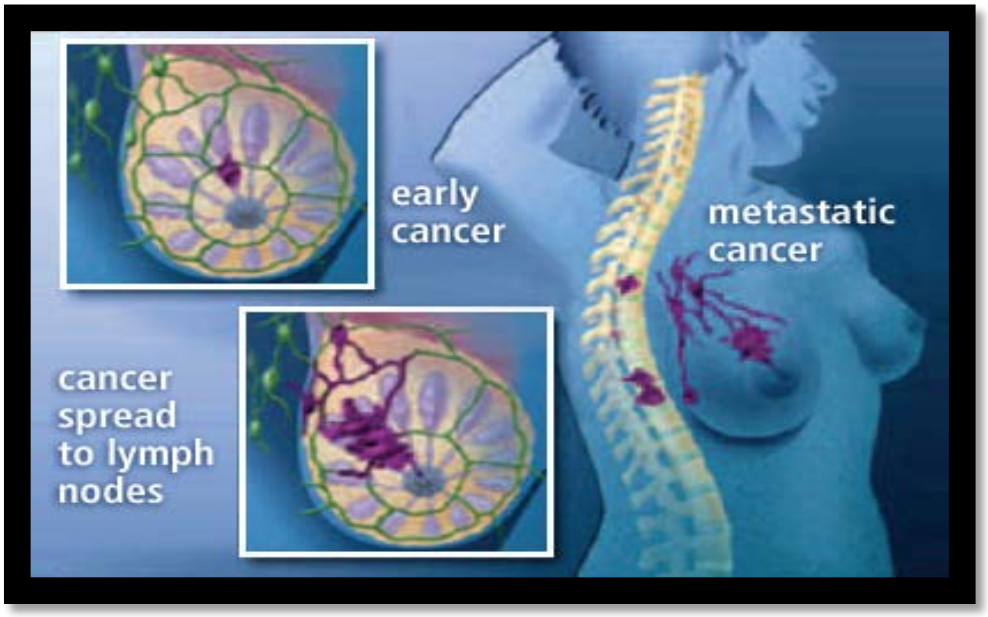

Figure (1-2) Showed Invasive and Metastasis of Breast Cancer (American Cancer Society 2006).

1.4. Possible Symptoms of Breast Cancer

In the early stages of breast cancer,the patient may not experience any obvious or painful symptoms. When experiencing symptoms, there will vary based on the size and location of the tumor in the breast (Osteen,2001; William,2007).

- painless lump in the breast
- palpable lymph node of axillary
- nipple retracting
- Discharge from the breast or nipple, usually bloody discharge from the nipple.
- Burning, itching or scaling of nipples.
- Pain or tenderness in the breast or underarm area.
- Change in the mental status, seizure, and neurological events can occur in the late stage of the disease due to involvement of brain or spinal cord.
- Jaundice may occur in the later stages of breast cancer due to the involvement of liver or surrounding structures.

1.5.Pathological staging of breast cancer

Breast cancer can be divided into two main groups: non-invasive or carcinoma in situ, and invasive carcinoma **(**Tabar *et al*., 1996**).**

1.Noninvasive breast cancer, or carcinoma *in situ*:- is a tumor that has not spread beyond the ducts or the lobules, depending on where it started (Bunnell,2003).

a) Ductal carcinoma *in situ* (DCIS) :- Cancer cells are located within a duct but are not found in surrounding fatty breast tissue. A form of DCIS that only involves the nipple (Paget's disease of the breast).

b) Lobular carcinoma *in situ* (LCIS) :- Abnormal cells grow within the lobules (milk-producing glands) but do not penetrate through the lobule walls. Most breast specialists do not consider LCIS a true breast cancer. LCIS is also called lobular neoplasia.

2. Invasive tumor:- has spread beyond where it began, and there are three different stages of invasiveness.

a) Localized stage:- cancers confined to the breast (Young, 2001).

b) Regional stage:- The tumor has spread to the tissue surrounding the breast or there are cancer cells within nearby lymph nodes. The more lymph nodes with cancer, the cancer may be more serious (Altman *et al* ., 2000).

c) Distant (advanced/metastatic) stage:- The tumor has spread away from the breast to other tissues in the body by lmphatic or blood (e.g., lung, liver, bone, or brain) (Altman *et al*., 2000).

1.6. Anatomical staging of breast cancer

Staging systems help describe the cancer, so that the doctor can decide what treatments are appropriate such as whether the tumor is operable (meaning that surgery should be done to remove the tumor). The TNM (Tumor, Nodal, and Metastasis) staging system is the most common method of staging breast cancer.

According to the TNM system, breast cancer is grouped into five stages from 0 to IV based on how large the tumor is, the tumor's nodal status (whether or not cancer cells have spread to the lymph nodes), and whether the tumor has spread (metastasis) (American Cancer Society, 2006). Terms "early" and "advanced" are sometimes used to describe tumors, but these terms may be used differently by different doctors. Generally, "early" or "early-stage" breast cancer means that the cancer has not spread beyond the breast or lymph nodes under the arm (known as axiliary lymph nodes).

Stage 0, I, and II, as well as some stage III cancers, are usually considered early-stage. Here are brief descriptions of each stage of breast cancer, according to the TNM system. (National Cancer Institute 2007).

Stage **0:-** is very early breast cancer. The cancer cells are still only in the duct or lobule where they began.

Stage **I:-** Tumor measures 2 cm (0.8 in.) or smaller in diameter and has not spread to lymph nodes in the armpit.

Stage **II:-** is divided into sub stages known as IIA and IIB (Singletary, 2002).

- **Stage IIA**: Either of the following characteristics is true of this stage of breast cancer:

1. The tumor is smaller than 2 cm (0.8 in.) in diameter and has spread to 1 to 3 lymph nodes under the arm on the same side as the breast cancer. Lymph nodes are not stuck to one another or to the surrounding tissues.

2. The umor is larger than 2 cm (0.8 in.) but smaller than 5 cm (2 in.) in diameter and has not spread to the axillary lymph nodes.

- **Stage IIB**: Either of the following characteristics is true of this stage of breast cancer:

1. The tumor is larger than 2 cm (0.8 in.) but smaller than 5 cm (2 in.) in diameter and has spread to 1 to 3 lymph nodes on the same side as the breast cancer. Lymph nodes are not stuck to one another or to the surrounding tissues.

2. The tumor is larger than 5 cm (2 in.) in diameter but has not spread to the lymph nodes under the arm.

- **Stage III:-** is divided into substages known as IIIA, IIIB, and IIIC (Bunnell,2003).
- **Stage IIIA**: Either the tumor or tumors measure larger than 5 cm (2 in.) in diameter with any spread to lymph nodes; it is smaller than 5 cm (2 in.) but the lymph nodes are stuck to one another or surrounding tissue; or it has spread to 4 to 9 lymph nodes.

- **Stage IIIB**: Breast cancers of any size have spread to the skin or chest wall.

- **Stage IIIC**: Breast cancers of any size have one of the following characteristics:

1. Breast cancer cells have spread to 10 or more lymph nodes under the arm.

2. Breast cancer cells are found in the lymph nodes under the arm and beneath the breast bone (internal mammary nodes).

3. Breast cancer cells are found in the lymph nodes above the collarbone (supraclavicular) or below the collarbon (infraclavicular).

Stage IV:- Cancer of any size has spread (metastasized) to distant sites, such as the bones or lungs, or to lymph nodes not near the breast.

1.7. Risk factors of breast cancer

Many of the known breast cancer risk factors, such as age, family history, menstrual history, genetic factors, menopause, and radiations, are not easily modifiable. However, other factors associated with increased breast cancer risk (use of hormone replacement therapy, alcohol consumption, smoking and breast feeding) are modifiable. Some risk factors directly increase lifetime exposure of breast tissue to circulating ovarian hormones (early menarche, late menopause, obesity, and hormone use), whereas others, such as higher socioeconomic status, are only correlates of reproductive behavior or other factors (Fentiman *et al* 2001; Chlebowaski *et al*., 2005).

1.7.1 Age

Age is the most influential risk factor for developing breast cancer. Women younger than age 40 accounted for only 4.7% of invasive breast cancer diagnoses and only 3.6% of in situ breast cancer diagnoses. Over 70% of all breast cancer diagnoses are made in women who are 50 or older (Osteen, 2001). The chance that a woman gets breast cancer increases with age. Statistically 1 in 8 Women in the United States will develop breast Cancer. This does not mean that a woman of any age has a 1 in 8 chance of developing breast cancer. The risk in a 20 year old female is extremely low (but not zero), but as she grows older, the chances of developing the disease substantially increase (Guembarovski *et al*., 2002). The age remain one of the single greatest risk factor for the development of new breast cancer with the estimated risk of new breast cancer at 1 in 14 for women aged 60-79

compared with 1 in 24 for women aged 40-59 and 1 in 228 women aged 39 and younger (Jemal *et al*., 2003).

1.7.2. Family history

Women with a relative who has had breast cancer are at higher risk of developing breast cancer themselves, particularly if it is a first-degree relative (mother, sister or daughter). That risk is further increased if a woman has multiple first-degree relatives who have had breast cancer or if she has a first-degree relative who developed breast cancer at a young age or in both breasts (Emery, 2001). In small groups of families, the patterns of breast cancer seem to be consistent with the known patterns of genetic inheritance. In less than 5% of breast cancer cases there is a fault in a gene that can be passed through families from one generation to the next. These faults can be inherited from either side of the family. The rare families with an inherited gene fault that predisposes to breast cancer tend to have a stronger family history with multiple women in several generations being diagnosed with breast cancer, often at a young age (Evans, 2002). Ovarian cancer and cancer of the fallopian tube (which joins the ovary to the womb) sometimes has an association with an inherited tendency to breast cancer. Genetic counseling and testing through a family cancer clinic may help families who suspect they may have an inherited gene fault (Lynch, 2008).

1.7.3. Genetic risk factors

Between 5% and 10% of breast cancers are inherited. Defects in one of several genes, especially BRCA1 or BRCA2, put women at greater risk of developing the disease (Jara *et al* ., 2005). Usually these genes help prevent cancer by making proteins that keep cells from growing abnormally but if

they have a mutation, the genes are not as effective at protecting women from cancer (DeMichele and Weber, 2000).

1.7.4. Radiation

Exposure to high doses of chest radiation (i.e. medical therapy for Hodgkin's disease), particularly during childhood, can greatly increase a woman's risk of developing breast cancer (Davis *et al*., 1994). Radiation causes breaks in DNA, and consequently mutations. Such mutations have been reported in the breast cancer gene BRCA1 (Miki *et al*., 1997). The latency period can be extremely long for people who have radiation exposure. Researchers have found that the age at which radiation was received is inversely related to the acquired risk. Thus, women who received radiation after their menopausal years incurred very little risk. Radiation exposure after the age of 40, however, seems to be of little danger (Boice, 2001).

1.7.5. Menopause and Menstrual history

Women who go through menopause after the age of 54 have a slightly higher risk of breast cancer than women who go through menopause at age 54 or younger. Their higher risk may be related to their higher lifetime exposure to estrogen and progesterone (Clemons *et al*., 2001). Breast cancer risk increases with early menarche and late menopause, and is reduced by early first full-term pregnancy (Chap *et al*., 2001). The pattern of menses following menarche also influences breast cancer risk. Among women who were pre-menopausal at the time of operation, a bilateral oophorectomy before the age of 50 years was associated with a 50% reduction in the risk of breast cancer whil oophorectomy after the age of 50 years in pre-menopausal

women or after a natural menopause was not associated with any reduction in risk (Catherine *et al*., 1997).

Other Risk Factors

1.7.6. Alcohol consumption

Many epidemiological studies spanning the past 20 years have shown an association between alcohol consumption and increased risk of breast cancer. Breast cancer risk is related to the amount of alcohol consumed (i.e. higher consumption of alcohol equals higher breast cancer risk. Alcohol consumption causes elevated serum levels of oestradiol, low levels of testosterone, follicle stimulatin hormone, luteinizing hormone and disruption of the normal feed back mechanisms (Dorgan *et al*., 2001). Both alcohol intake and elevation of estrogen level would argue for a substantial increase in breast cancer risk (Singletary *et al*., 2001).

1.6.7. Smoking

A recent study suggested that women who were exposed to cigarette smoke (both active and passive smoking) were indeed at higher risk of Breast cancer (Kropp *et al*., 2002). In Danish women interviewed at the time of mammography, smoking for more than 30 years was associated with a 60% higher risk of breast cancer and age of onset at an average of eighty years earlier, when compared with non-smokers (Gupta *et al*., 2001). Although the association between smoking and breast cancer risk is not clearly established, smoking is strongly discouraged because of its known impact on a person’s risk of heart disease and lung cancer.

1.7.8. Hormone Replacement Therapy

A study conducted by the Women's Health Initiative (WHI) showed that women in the study population who took hormone replacement therapy (combined estrogen and progestin) had a 26% increased risk (relative to an average woman) of invasive breast cancer after four to five years of therapy (Stefanick *et al*., 2004). Short term estrogen replacement therapy appears to be safe in evaluating the potential risk of breast cancer from estrogen therapy,but the proven benefits in reducing osteoporosis and subsequent fracture and lowering the risk of coronary artery disease, must be taken into account (Seth *et al*., 2000).

1.7.9. Breast feeding

Women who have breastfed for at least 12 months are at lower risk for breast cancer. In a reanalysis of data from 47 epidemiological studies in 30 countries, breastfeeding for a total of 12 months or more in their lifetime (i.e., does not need to be consecutive) was associated with a lower risk of breast cancer (i.e., a 4.3% decrease in relative risk of breast cancer for every 12 months of breastfeeding). In a Swedish study, breastfeeding for more than one year was associated with lower risk of breast cancer among women with BRCA1 mutations, but not among women with BRCA2 mutations. (Collaborative Group on Hormonal Factors in Breast Cancer, 2002).

1.8. Hormones and Breast cancer

Female hormones that affect growth of the mammary gland are potential risk factors for breast cancer (Corwin, 2008).

Hormones act by binding to specific receptors of target cells to form a complex that elicits a cellular response. Only the target tissue will express the receptor for a given hormone and be able to respond to it. Hormone receptors may be located on the surface of the cell or within the cell (Platet *et al*., 2004).

1.8.1. Estrogen

Estrogen is a hormone acting as a chemical messenger in the body. It is important for normal sexual development and is essential for the normal functioning of the female organs needed for childbearing such as the ovaries and uterus. Estrogen helps control a woman's menstrual cycle. It is important for the normal development of the breast during the childbearing years from puberty to menopause; Organs called the Ovaries produce estrogen and the studies have shown that women who had their ovaries removed early in life have a very low incidence of breast cancer (Key *et al*., 1998). Many researchers have examined the possible relationship between exposure to estrogen and breast cancer risk. Dorgan *et al*., (1996) showed that women who had been treated for breast cancer, and who had higher levels of estrogen in their bodies, had a return of the disease sooner than women treated for breast cancer and who had lower levels of estrogen. Estrogens, especially estradiol and estrone, stimulate proliferation of breast tissue and breast cancer cells (Goldfie *et al*., 1997).

Estradiol, the most active estrogen produced by the ovary, is synthesized from androgens by the enzyme aromatase. Estrogen is influenced by several environmental factors (Miller *et al*., 1990). On the other hand, estrogen is linked with both environmental as well as genetic factors. In the same manner, breast cancer is considered as the outcome of a complex interplay amongst genetic, hormonal, and environmental factors.

In addition to modulating growth factor genes, estrogen may increase the production of proteases such as pro-cathepsin D which can enhance the invasiveness of tumor cells (Garcia *et al*., 2001). After menopause, these extra ovarian sources of estrogen may play an important pathophysiological role since two-thirds of breast cancer occur during the postmenopausal period, and about 33- 50% of human breast cancers respond to hormonal treatment (Simpson *et al*., 2001).

1.8.2. Progestron

Progesterone is one of two main reproductive hormone groups, the other being the estrogens, made by the ovaries of menstruating women (Arpino *et al*., 2005). It primarily a hormone of fertility and pregnancy and it effects on breast epithelium (Colditz *et al*., 2004).

The three major functions of progesterone in the body are:

- To promote the survival and development of the embryo and fetus
- To provide a broad range of core biologic effects
- To act as a precursor (building block) of other steroid hormones

Progesterone is a precursor (or building block) to many other steroid hormones such as cortisol, testosterone and estrogen (estriol, estradiol, estrone). Because it is a modulator, its use can greatly enhance overall hormonal balance. Progesterone supplementation will stimulate bone building and help protect against osteoporosis, not overlooking the numerous positive roles it plays in the body. For women who suffer hormonal imbalance but are not necessarily menopausal, progesterone is equally important (Arpino *et al*., 2005). possibly progesterone may stimulate breast cancers that have already begun to grow (promoter effects) rather than cause the disease. Tumor promoters do not by themselves cause mutations

but have the effect of stimulating the cell division that enables mutant cells to propagate. Since most mutations require the effects of a promoter to cause cancer, removal of promoters from the environment may help prevent the cancers from developing (Migliaccio *et al*., 2008).

1.9. Plasma lipids effect on breast cancer

Lipids are major cell membrane components essential for various biological functions including cell growth and division of normal and malignant tissues. Usefulness of variations in tissue, blood cholesterol levels in diagnosis and treatment of various diseases has been studied by several workers. Although, its prime role in pathogenesis of coronary heart disease has been consistently found, researchers have reported association of plasma, serum lipids and lipoproteins with different cancers (Allampallam, 2002). The alterations in the circulatory cholesterol, triglyceride and high density lipids levels have been found to be associated with etiology of breast cancer (Forones *et al*., 1998). Epidemiological, experimental, and clinical data strongly support the possibility that breast cancer will be affected by lipid profiles in postmenopausal women (Gail *et al*., 1999). Serum triglycerides (TG), high-density lipoprotein cholesterol (HDL), total cholesterol (TC) were measured at baseline and to assess the impact of breast cancer on the patients and determined the degree of postmenopausal metastatic breast cancer (Paridaens *et al*., 2000). In some malignant diseases, blood cholesterol, HDL and TG undergoes early and significant changes. Low levels of cholesterol in the proliferating tissues and in blood compartments could be due to the process of carcinogenesis (Eichholzer *et al*., 2000) . Accordingly, based on the fact that the normal variation in plasma lipid could influence the subsequent risk of breast cancer in postmenopausal women, it is reasonable to postulate that profound TC, TG,

HDL may be associated with a significantly increased risk of breast cancer (Lundgren *et al*., 1996).

Vainio *et al*., (2000) was hypothesized that HDL-C, TC, TG as an important components of the metabolic syndrome, may influence the risk of breast cancer and these factors may be an important clinical markers of breast cancer risk that may be more pronounced in women with positive energy balance (i.e., with long-term excessive energy intake relative to requirements, as reflected in a high BMI) and their age more than 40 years.

1.10. Molecular Pathogenesis of Breast Cancer

The molecular pathogenesis of breast cancer involves genetic alterations of breast epithelial cell DNA resulting in progressively more invasive and malignant somatic cells (Wazer *et al*., 1999). The process is probably initiated by a variety of carcinogens including chemical, radiation (Mattsson *et al*., 1993) and possibly retroviruses (Al-Sumidaie *et al*., 1988) and it can be promoted by many physiologic and environmental factors. Breast cancer usually arises in women a Sporadic event. It develops in genetically altered cells of ductal lobular unit, mainly through a multistep series of genetic alterations. The most important and best-established genes involved in this Process are the BRCA1 gene and the p53 gene found on chromosome17 and the BRCA2 gene found on chromosome 13 (Linnea *et al*., 2001).

1.10.1 BRCA1 & BRCA 2

BRCA mutation runs a greater risk of developing breast cancer and significantly higher risk in women at age > 40 years old. In normal cells, BRCA1 and BRCA2 help ensure the stability of the cell's genetic material

(DNA) and help prevent uncontrolled cell growth. Mutation of these genes has been linked to the development of hereditary breast cancer (Kadouri *et al*., 2007). Both genes are large and multi-exonic: the coding region of BRCA 1 contains 5592 base pairs distributed over 22 coding exons and BRCA 2 contains 10254 base pairs of coding sequence within 26 exons shown as figure (1-4) (Tavtigian *et al*., 1996).

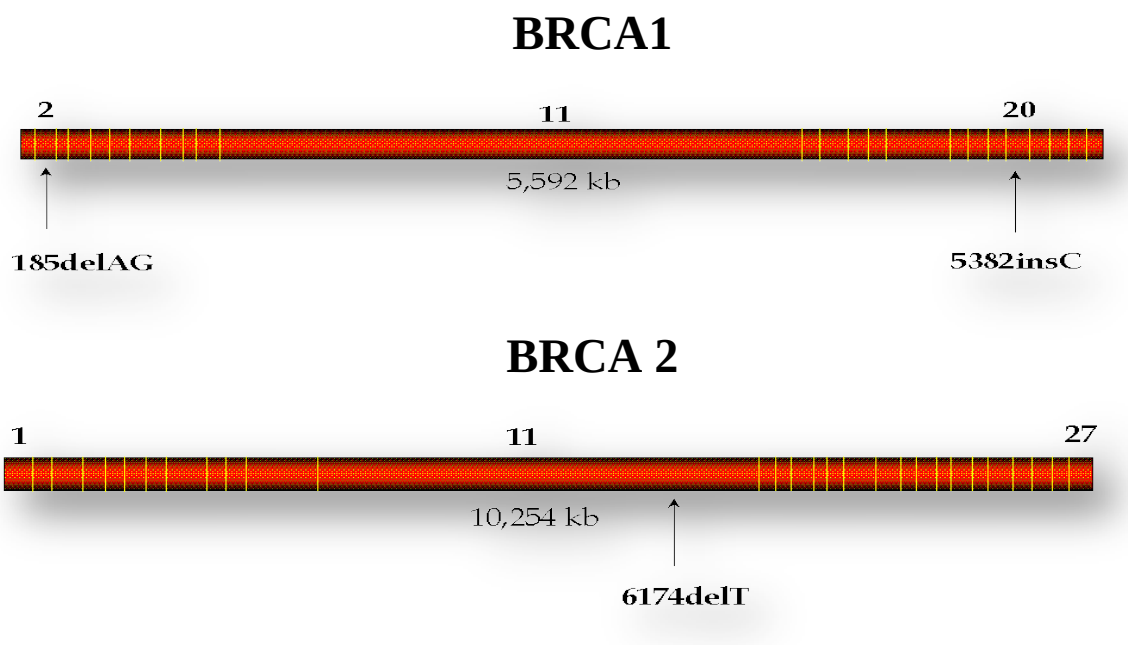

Figure (1-3) diagram of structure and location of axons of BRC1 and BRCA2.

BRCA1 (breast cancer 1, early onset) is a human gene, some mutations of which are associated with a significant increase in the risk of breast cancer, as well as other cancers. BRCA1 belongs to a class of genes known as tumor suppressors, which maintains genomic integrity to prevent dangerous genetic changes. The multifactorial BRCA1 protein product is involved in DNA damage repair especially error-free repair of DNA double strand breaks, ubiquitination, transcriptional regulation as well as other functions

(Friedenson, 2007) . The BRCA1 protein is directly involved in the repair of damaged DNA. In the nucleus of many types of normal cells, the BRCA1 protein is thought to interact indirectly with RAD51 during repair of DNA double-strand breaks (Boulton, 2006) . These breaks can be caused by natural radiation or other exposures, but also occur when chromosomes exchange genetic material (homologous recombination, e.g. "crossing over" during meiosis).

The BRCA2 gene (Breast Cancer Type 2 susceptibility protein) is located on the long (q) arm of chromosome 13 at position 12.3 (13q12.3), from base pair 31,787,616 to base pair 31,871,804 (Yoshida, 2004). BRCA2 is a protein that in humans is encoded by the BRCA2 gene. BRCA2 belongs to the tumor suppressor gene family and the protein encoded by this gene is involved in the repair of chromosomal damage with an important role in the error-free repair of DNA double strand breaks. Certain variations of the BRCA2 gene cause an increased risk for breast cancer. Researchers have identified hundreds of mutations in the BRCA2 gene, many of which cause an increased risk of cancer. BRCA2 mutations are usually insertions or deletions of a small number of DNA base pairs (the building material of chromosomes) in the gene. As a result of these mutations, the protein product of the BRCA2 gene is abnormal and does not function properly. Researchers believe that the defective BRCA2 protein is unable to help fix mutations that occur in other genes. As a result, mutations build up and can cause cells to divide in an uncontrolled way and form a tumor (Dong, 2007).

The BRCA2 protein, which has a function similar to that of BRCA1, also interacts with the RAD51 protein. By influencing DNA damage repair, these three proteins play a role in maintaining the stability of the human genome.

Certain variations of the BRCA1 gene lead to an increased risk for breast cancer. Researchers have identified hundreds of mutations in the BRCA1 gene, many of which are associated with an increased risk of cancer. 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, 2008) .

1.10.2. p53

p53 is encoded by the Tp53 gene. Located at 17p13, this contains 11 exons spanning 20 kb. It belongs to a family of highly conserved genes that also includes TP63 and TP73, encoding p63 and p73 respectively (Savkur & Burris 2004). In normal cells not exposed to stress, the level and activity of p53 are very low. Upon stress, p53 is activated through a series of post-translational modifications and becomes able to bind to specific DNA sequences. The p53 recognition sequence is very loose and has been found in several hundred genes that are differentially modulated (induced or repressed) depending on the cell type, the nature of stress and the extent of damage. At low cellular levels, p53 modulates only a subset of the genes regulated at higher levels. The kinetics of target gene modulation may also vary. In a study with a micro-array carrying 6000 capture sequences, 107 genes were found to be induced and 54 genes were repressed by p53 (Zhao *et al.*, 2006). The central core region of p53 is of key importance in regulating apoptotic function, either transcription-dependent or -independent, as supported by the number of mutations affecting this region in apoptosis-deficient p53 cells. In addition to inducing genes that drive apoptosis, p53 can also activate the expression of genes that inhibit survival signaling (such as PTEN) or inhibit inhibitors of apoptosis (such as BIRC5).

Deletion of this region leads to a complete loss of the apoptotic activity of p53 (Yu & Zhang 2005). P53 mutations may be observed in the rare familial autosomal Li Fraumeni syndrome. It is characterized by a high incidence of multiple early cancers, including breast tumors. Other hereditary breast cancers may be due to mutations in genes coding for p53 modulator proteins. A significant proportion of these cancers have been associated with mutations of BRCA1. BRCA1 may interact with p53 and has been viewed as a 'scaffold' for p53 response (Hohenstein & Giles 2003).

Of interest, BRCA1 tumors often express Tp53 mutations, but it remains to be established if this reflects the need for p53 inactivation for the development of BRCA1 tumors to occur, or rather if the loss of BRCA1-associated DNA repair properties may explain, at least partly, the high frequency of Tp53 mutations (Lacroix & Leclercq 2005).

1.10.3 Human epithelial receptor 2

(HER-2/neu): a member of the class I growth factor-receptor tyrosine kinase family, the genetic abnormality is not inherited but acquired (Chap *et al*., 2001). The HER-2 (human epithelial receptor 2, also known as HER-2/neu or erbB-2) gene is located on chromosome 17q and encodes a 185-kDa transmembrane tyrosine kinase growth factor receptor .The HER-2 is another gene found on the surface of cell that plays a key role in regulating cell growth. This over-expression of HER-2 increases cell growth and reproduction, often resulting in more aggressive tumor (Raj *et al*., 2001). Women with breast cancer that over expresses human epidermal growth factor receptor type 2 (HER2, also referred to as HER2/neu) are at greater risk for disease progression and death than women whose tumors do not over express HER2 (Vogel, 2008). Eighteen to twenty percent of invasive breast cancers overexpress HER2, which has both prognostic and predictive

implications. Prior to the routine use of adjuvant trastuzumab therapy, HER2 overexpression was associated with a more aggressive tumor phenotype and worse prognosis (higher rate of recurrence and mortality) especially in patients who do not receive adjuvant chemotherapy. Additionally, HER2 status has been shown to be predictive for response to certain chemotherapeutic agents (ie, doxorubicin, and HER2 targeted therapies, trastuzumab, a monoclonal antibody, and lapatinib) directed specifically to the HER2 receptor (Park, 2008).

1.11. Detection Breast cancer by PCR technique

PCR (polymerase chain reaction) is the patented technique for synthesizing strands of DNA in large number of copies, enabling the material produced to be utilized both for research and practical application purposes such as examining genetic alterations in tumor samples (Sigrid *et al*., 1994). PCR technique has facilitated identification of many defects, including a variety of nonrandom genetic lesions in breast cancer. Identification of those genetic alterations may become an important step in the diagnosis of breast carcinoma and may eventually allow tumor sub typing beyond standard histological and clinical groups (Agnishwar, 2008). Ultimately, the hope for this technology is that it will improve ability to determine prognosis and optional therapeutic intervention for individual patients and will lead to a better understanding of human breast carcinoma. PCR-based techniques may hold great promise for distinguishing microscopically similar yet genetically and biologically distinct subsets of breast lesions (Thor, 1998). Complementary oligodeoxyribonucleotide primers and the polymerase chain reaction are used to generate two DNA fragments having overlapping ends. These fragments are combined in a subsequent 'fusion' reaction in which the overlapping ends anneal, allowing

the 3' overlap of each strand to serve as a primer for the 3' extension of the complementary strand. The resulting fusion product is amplified further by PCR. Specific alterations in the nucleotide sequence can be introduced by incorporating nucleotide changes into the overlapping oligo primers (Hunt *et al*.,2004). This method improvement more efficient for screening of specific characterized mutations because it is much faster, simpler and approaches 100% efficiency in the detection of mutant product.

MS-PCR-based technique is specific type of PCR technique used to detect both normal and mutant alleles by amplified them in the same reaction tube, using different length allele-specific primers (Teng *et al*., 2006). Furthermore the allele-specific primers introduce additional deliberate differences into the allelic PCR-products that drastically reduce cross reactions in subsequent cycles. Subsequent identification of the PCR-products is done by gel electrophoresis and shows at least one of the two allelic products. MS-PCR provides a within-assay quality control for the exclusion of false negative results (Rust, 2008).

Chapter Two

Materials

And

Methods

Chapter Two

Materials and Methods

2.1. Materials

2.1.1. Subjects

Twenty Five fresh biopsy from breast cancer women their age about 40 years were included in this study (their main age 50 years old). Samples were collected prospectively at Al-Kadhymia Teaching Hospital through the period from November 2007-March 2009. The main data and parameter include in our study: patient's age, family history of breast cancer and another cancer, age at menarche and blood group. The breast cancer women were interviewed and questioned according to special form (Appendix -I). Confirmatory diagnosis of breast cancer and it differentiation were identified by histopathological examinations, also the study included ten apparently normal female individuals with different menstrual status and with satisfactory reproductive history; all were married and at least have one child with history of previous lactation.

2.1.2. Equipments

The equipments and their sources are given below

Equipment	Company	Country
Automatic Micropipette	Slamed	England
Centrifuge (Minispin)	Eppendorf	Germany
Digital camera	Sony	Japan
Distillator	GFL	Germany
Electrophoresis power supply	Amersham	Sweden
Eppendorf (1.5,o.25ml)	Clay Adams	Germany
Flasks	Simax	USA
Gel electrophoresis apparatus	Amersham	Sweden
Hood	Telestar	Spain
Magnetic stirrer	Corning	USA
Microfug (mix 12000-14000 rpm)	Beckman coulter	Germany
Oven shaker	Thermo electron	USA
Refrigerator	Hitachi	Japan
UV light transillminator	Ultraviolet Products institute	USA
Vortex	Clay adams	Germany

2.1.3. Chemicals

The major chemicals and biological used and their sources are given below

Chemicals	Source	Country
Absolute ethanol	Fluka	Germany
Agarose	Sigma	Germany
Bromophenol blue	BDH	England
DNA kit	Promega	USA
DNA ladder Marker (1500bp)	___	_____
Ethidium bromide	BDH	_____
High Lipid Density	linear chemical	Spain
Master mix	Alpha DNA	Canada
Primers	Promega	_____
RIA Estradiol Kit	Beckman counter	Germany
Total cholesterol	linear chemical	Spain
Triglycerides	___	___

2.1.4. Reagents

The following reagents were prepared according to Sambrooke and Russell (2001).

2.1.4.1. Isopropanol

Absolute isopropanol prepared according to Sambrooke and Russell (2001) and stored at 4ºC.

2.1.4.2. 70% Ethanol

It was prepared by mixing 70 ml of absolute ethanol with 30 ml distilled water and stored 4 ºC

2.1.5. RIA (radio immuno assay) estradiol kit

REAGENT
Anti-estradiol antibody coated tubes
^{125}I-labeled estradiol
Tracer buffer
Calibrators
Control serum

2. 1.6. Genomic DNA isolation kit

The genomic DNA isolation kit was provided by Promega Company / USA. The components of the kit are the following:

1- Cell lysis solution

2-Nuclei lysis solution

3-Protein precipitation solution

4-DNA rehydration solution

5-RNAse solution

2.1.7. Solution and buffers in agarose gel electrophoresis (Sambrooke and Russell, 2001)

2.1.7.1. 1X TBE buffer (Tris/Borate/EDTA) electrophoresis buffer

Tris – base	54g
Boric acid	27.5g
EDTA(0.5M,PH 8.0)	20ml
Distilled water	980ml

2.1.7.2. Ethidium Bromide (10mg/ml)

One gram of Ethidium bromide was added to 100 ml of distilled water and stirred on a magnetic stirrer for several hours to ensure that the dye was dissolved .The container was wrapped with aluminum foil and kept in a dark bottle and stored at room temperature**.**

2.1.7.3. 6X loading buffer:

Glycerol	50 %
Tris-base pH(8)	0.025 M
Bromocresol purple	0.25%

2.2. Methods

2.2.1. Samples Collection

A. Blood Sampling:

Three to five ml of blood was collected in plain tubes from 10 patients and from the 10 control individuals. Blood samples were drawn

at the morning between 9 and 9:30 am from cubital vein. Blood samples were centrifuged at 3000 rpm for 2-5 minutes then serum was separated to be stored immediately in the process of hormonal assay.

B. Tissue Sampling:

From each patient one g fresh tissue was collected in container tubes with normal saline. All samples were obtained after informed consent of the participants prior to their inclusion in the study. A structured questionnaire was used to elicit detailed information on age, age at menarche, blood group and family history of breast cancer and another type of cancer (appendix-1).

2.2.2. Laboratory work

A. Hormonal Study of Blood Samples

Assay procedure of Estradiol by RIA Estradiol Kit

Step 1 (addition)

To coated tubes, 100µl of calibrator, control or sample were sequentially added and then 500 µl of tracer was added, and mixed briefly.

Step2 (Incubation)

The mix was incubated for 3 hours at 18-25 ºC with shaking.

Step 3(counting)

The contents of the tube were aspirated carefully, placed in Gamma counter and then bound cpm and total cpm were counted.

B. Lipid Profile Study of Blood Samples by Linear Chemical/Spain

1. Total Cholesterol

Principle:-

This method for measurement of total cholesterol in serum involves in the use of three enzymes: cholesterol esterase (CE), cholesterol oxide the (CO) and peroxidase (POD). In presences of the former the mixture of phenol and 4-aminoantipyrine (4-AA) are condensed by hydrogen peroxide to form a quinoneimine dye proportional to the concentration of cholesterol in the samples.

Cholesterol esters $\longrightarrow$ **Cholesterol + Fatty acids**

Cholesterol + O_2 $\longrightarrow$ **Cholestenone + H_2O_2**

4-AA + Phenol $\longrightarrow$ **Quinoneimine + $4H_2O$**

- Reagents and samples were bringing to room temperature.
- Reagents and samples were Pipette into labeled tubes:

Tubes	Blank	Samples	Standard
Monoreagents	1.0ml	1.0ml	1.0ml
Samples	-	10µl	-
Standers	-	-	10µl

- The tubes were mixed and let them stand 10 minutes at room temperature or 5 minutes at 37 ºC.

- The absorbance (A) of the samples was read and the standard at 500 nm against the reagent blank.
- The color was stable for at least 30 minutes protected from light.

$$\frac{A_{sample}}{A_{stander}} \times C_{stander} = \text{mg/dL total cholesterol}$$

2. Triglycerides

Principle:-

The method is based on the enzymatic hydrolysis of serum or plasma triglyceride to glycerol and free fatty acids (FFA) by lipoprotein lipase (LPL). The glycerol is phosphorylated by adenosine triphsphate (ATP) in the presence of glycerolkinase (GK) to form glycerol-3-phosphate (G-3-P) and adenosine diphsphate (ADP). G-3-P is oxidized by glycerophosphate oxidase (GPO) to form dihydroxyacetone phosphate (DHAP) and hydrogen peroxide. A red chromogen is produced by the peroxidaes (POD) catalyzed coupling of 4-aminoantipyrine (4-AA) and phenol with hydrogen peroxide (H_2O_2), proportional to the concentration of triglyceride in the sample.

$$\text{Triglycerides} + 3H_2O \xrightarrow{LPL} \text{Glycerol} + 3\text{FFA}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{GK} \text{Glycerol- 3-P} + \text{ADP}$$

$$\text{Glycerol- 3-P} \xrightarrow{GPO} \text{DHAP} + H_2O_2$$

$$\text{4-AA+ 4 Phenol} \xrightarrow[\text{POD}]{H_2O_2} \text{Quinoneimine} + H_2O$$

- Reagents and samples were bringing to room temperature.
- Reagents and samples were Pipette into labeled tubes:

Tubes	Blank	Samples	Standard
Monoreagents	1.0ml	1.0ml	1.0ml
Samples	-	10µl	-
Standers	-	-	10µl

- The tubes were mixed and let them stand 15 minutes at room temperature (16-25ºC) or 5 minutes at 37 ºC.
- The absorbance (A) of the samples was read and the standard at 500 nm against the reagent blank.
- The color was stable for at least 1 hours protected from light

$$\frac{A_{sample}}{A_{stander}} \times C_{stander} = \text{mg/dL Triglycerides}$$

3. High Lipid Density Lipoproteins

- Reagents and samples were bringing to room temperature.
- Reagents and samples were Pipette into labeled tubes:

Tubes	Blank	Samples	Standard
Monoreagents	1.0ml	1.0ml	1.0ml
Samples	-	10µl	-
Standers	-	-	10µl

- The tubes were mixed and let them stand 15 minutes at room temperature (16-25ºC) or 5 minutes at 37 ºC.
- The absorbance (A) of the samples was read and the standard at 500 nm against the reagent blank.
- The color was stable for at least 30 minuet protected from light**.**

$$\frac{A_{sample}}{A_{stander}} \times C_{stander} = \text{mg/dL C-HDL}$$

2.2.3. Molecular Study of Tissue Samples

2.2.3.1. Isolation of genomic DNA by using Promega kit :

- Six hundred µl of Nuclei Lysis Solution was added to a 15 ml centrifuge tube, and chilled on ice.
- Ten to twenty mg of fresh or thawed tissue was added to cooled. Nuclei Lysis Solution and homogenized manually for 10 seconds using small homogenizer. The lysate was transferred to a 1.5ml micro centrifuge tube.

- The lysate was incubated at 65˚C for 15-30 minutes.
- Three µl of RNAse Solution was added to the nuclear lysate and the sample was mixed by inverting the tube 2-5 times. The mixture was incubated for 15-30 minutes at 37˚C. The sample was allowed to cool at room temperature for 5 minutes before proceeding.
- Two hundred µl of protein precipitation solution was added to the room temperature sample and vortexes at high speed for 20 seconds, the sample was chilled on ice for 5 minutes.
- The sample was centrifuged for 4 min at 13,000 rpm. The precipitation protein form tight white pellet.
- The supernatant containing the DNA was carefully removed and transferred to clean 1.5ml centrifuge tube containing 600µl of room temperature isopropanol.
- The solution was gently mixed by inversion until the white thread like strands of DNA form visible mass.
- The solution was centrifuged for one minute at 13, 000-16, 000 rpm at room temperature. The DNA was visible as a small white pellet. The supernatant was carefully decanted.
- Six hundred µl at room temperature 7o% ethanol was added, and the tube was gently inverted several times to wash the DNA .The solution was centrifuged for 1 min at 13,000 rpm at room temperature.
- The ethanol was carefully spirited using either a drawn Pasteur pipette or sequences pipette tip. The DNA pellet was very losing at this point, and care must be used to avoid aspirating the pellet into the pipette.
- The tube was inverted on clean filter paper, and the pellet was air-dried for 10-15 min.

- One hundred µl of rehydration solution was added and the DNA was rehydrated by incubing at 65°C for 1hr. The solution was mixed by gently tapping the tube. Alternatively, the DNA was rehydrated by incubating the solution over night at room temperature or at 4 ˚C.
- The DNA was stored at 2-8˚C.

2.2.3.2. DNA purity and concentration

Measurement of the DNA purity and concentration were carried out by using spectrophotometer which designed for such measurement. Moreover, estimation of DNA purity and concentration were determined according to the procedure reported by Sambrooke and Russell (2001).

1. Estimation of DNA concentration (Sambrooke *et al.*, 1989)

DNA sample was diluted with TE buffer (pH 7.6) to 1:100 and mixed thoroughly, and then the optical density (O.D) was measured in UV spectrophotometer at wavelength 260 nm. The concentration of the DNA in µg/ml of sample would be:

DNA concentration (µg/µl) = ($O.D_{260}$ X 100 X 50 µg/ml) /1000

Theoretically, O.D value of one is corresponding to approximately (50 µg/ml) for double stand DNA.

2. Estimation of DNA purity

The ratio between the reading at 260 nm and 280 nm provides an estimate of purity of nucleic acid, and was measured as following:-

DNA purity = $O.D_{260}$ / $O.D_{280}$

Pure preparation of DNA samples has ($O.D_{260}$ / $O.D_{280}$) values of 1.7 and 1.9 (Manchester, 1995).

2.2.4. Agarose gel electrophoresis

1. Reagents:

- Agarose
- 1 X TBE buffer.
- Bromophenol blue in 1% glycerol.
- DNA marker.
- Ethidium bromide (10 mg / ml).

2. Agarose gel was prepared according to Sambrooke *et al.*, (1989)

❖ **Preparation of agarose gel (0.8%)**

- Fifty ml of 1 X TBE (PH 8.0) was added to a beaker.
- 0.4 gm agarose was added to the buffer.
- The solution was heated to boiling (using water bath)
- The solution was allowed to cool down at 50-60°C.

❖ **Casting of the horizontal agarose gel**

- The gel was assembled to casting tray and the comb was positioned at one end of the tray.

- The agarose solution was poured into the gel tray after both edges were sealed with cellophane tapes and the agarose was allowed to be gel at room temperature for 30 minutes.
- The comb was carefully removed and the gel placed in electrophoresis chamber.
- The chamber was filled with TBE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel.

❖ **Loading and running DNA in agarose gel**

- DNA was mixed with loading buffer (ratio 3:1) and loaded in the wells of the agarose gel.
- The cathode was connected to the well side of the unit and the anode to the other side.
- The gel was run at 70 volt. Until the loading dye migrated to the suitable distance from the well.
- The DNA was observed by staining the gel with Ethidium bromide and viewed under UV transilluminator.

2.2.5. Mutagenically separated Polymerase Chain Reaction (PCR) amplification

The following chemicals were used for MS-PCR amplification (pak *et al*., 2008).

1) 1X PCR Master Mix

It was provided by Promega Company / U.S.A. with following composition:-

- Taq DNA polymerase.
- $MgCl_2$.
- dNTPs (dAtp, dCTP, dGTP , dTTP).

2) Primers

In this study describe a simple and rapid method for simultaneous detection of three common mutation 185delAG and 5382insC in BRCA1 and 6174delT in BRCA2. DNA was extracted from fresh biopsy tissue as described previously. Allele-specific oligonucleotide primers were designed on the basis of published BRCA1 BRCA2 sequences. For each mutation three primers (one common, one specific for the mutant and one specific for the wild type) were used. The competing mutant and wild type were designed to differ by 20 bp in size, allowing easy detection of PCR products by routine electrophoresis. The mutant and wild type primers both contain mismatched base sequence near 3' end. In the early cycles of amplification, the mismatched sequences generate mutagenized PCR products that are refractory to cross amplification by the competing primer, thereby ensuring specificity of the reaction. The mutant primer also incorporates two additional mismatched bases at two contiguous positions corresponding to the 5' end of the wild type primer. During the final cycles of PCR reaction, heteroduplexes may be formed the short and long products, but the contiguous mutagenized sequences in long product prevent filling up of the short product by using the long strand as template. As a result, the mutant and wild type products are separated mutagenically (pak *et al*., 2008). The primer sequences and sizes of corresponding amplicons are shown in table (2-1).

Table (2-1) Shown the Primers Sequences and Their Size of Amplicon

Primer		Sequences	Size of amplicon
1*BRCA1 185del AG	P1	5'GGTTGGCAGCAATATGTGAA'3	
	P2	5'GCTGACTTACCAGATGGGACTCTC '3	335bp
	P3	5'CCCAAATTAATCACTCTTGTCGTGACTTACCAGATGGGACAGTA'3	354bp
2*BRCA1 538 insC	P4	5'GACGGGAATCCAAATTACACAG'3	
	P5	5'AAAGCGAGCAAGAGAATCGCA'3	271bp
	P6	5'AATCGAAGAAACCACCAAAGTCCTTAGCGAGCAAGAGAATCACC3	295bp
3*BRCA2 6174del T	P7	5'AGCTGGTCTGAATGTTCGTTACT '3	
	P8	5'GTGGGATTTTTAGCACAGCTAGT '3	151bp
	P9	5'CAGTCTCATCTGCAAATACTTCAGGGATTTTTAGCACAGCATGG'3	171bp

P1= common forward P2= wild- type reverse P3= mutant reverse

P4= common reverse P5= wild-type forward P6= mutant forward

p7= common reverse p8= wild-type forward P9= mutant forward

2.2.6. PCR Reaction

The reaction mixture of MS-PCR was prepared according to the addition order shown in tables (2-2, 2-3, 2-4). The reaction mixture samples were mixed gently by vortex and centrifuge at 1300 rpm for few seconds to collect all drops to the bottom of tubes. The tubes were then placed in appollo thermal cycler (with heating lid) to carry out implication. The implications were run according to the program shown in tables. Twenty micro liter of amplified DNA was drawn into another tube and analyzed by agarose electrophoresis (pak *et al*., 2008).

1. Reagents were used in MS-PCR (25µl) at final concentration:

- To a 25 µl PCR tube, 4µl DNA was utilized.
- A 30 picomoles of primers forward and reverse (2µl for each), were added to the tubes, then 2.5 D.W was added (for each tube).
- Finally, 12.5µl master mixes (Promega Co.) were added. The BRCA1 and BRCA2 genotypes were analyzed by PCR; Genomic DNA was amplified by using 9 sets of primers (Table 2-2, 2-3, 2-4).

Table (2-2) The Reaction mix (25µl) for BRCA1 185delAG mutation

Addition order	Chemical	Volume	Concentration
1-	PCR Master Mix	12.5µl	2x
2-	P1	2 µl	30 pmol/ml
3-	P2	2 µl	30 pmol/ml
4-	P3	2 µl	30 pmol/ml
5-	DNA	4 µl	50 ng/µl
6-	D.W	2.5 µl	-
Not :- final volume =25 µl			

P1= common forward P2 = wild-type reverse P3 = mutant reverse

Table (2-3) The Reaction mix (25µl) for BRCA1 5382insC mutation

Addition order	Chemical	Volume	Concentration
1-	PCR Master Mix	12.5µl	2x
2-	P4	2 µl	30 pmol/ml
3-	P5	2 µl	30 pmol/ml
4-	P6	2 µl	30 pmol/ml
5-	DNA	4 µl	50 ng/µl
6-	D.W	2.5 µl	-
Not :- final volume =25 µl			

p4 = common reverse p5 = wild-type forward p6 = mutant forward

Table (2-4) The Reaction mix (25µl) for BRCA26174delT mutation

Addition order	Chemical	Volume	Concentration
1-	PCR Master Mix	12.5µl	2x
2-	P7	2 µl	30 pmol/ml
3-	P8	2 µl	30 pmol/ml
4-	P9	2 µl	30 pmol/ml
5-	DNA	4 µl	50 ng/µl
6-	D.W	2.5 µl	-
Not :- final volume =25 µl			

p7 = common reverse p8 = wild-type forward p9 = mutant forward

2. Primers and PCR condition:

The PCR products were subjected to electrophoresis on 2% agarose gel stained with Ethidium bromide.

The presence of bands of 354, 295 bps and 171 was indicative of the BRCA1 and BRCA2 genotypes whereas the absence indicated 335bp, 271bp and 151bp for these genes. A negative control without template DNA was used in each run.

Table (2-5): PCR conditions for BRCA1 and BRCA2 genes.

No.	Steps	Temperature	Time	No. of cycles
I	Denaturation	94ºC	15s	**1**
II	Annealing	57 ºC	15s	**1**
III	Extension	7 2ºC	30s	**1**
IV	Extension	72 ºC	15min	**1**
Total number of cycles				**35**

Because of the low quality and the low size of the products, another low melting agarose gel with the same concentrations (2%) was utilized. Furthermore, a new electrophoresis chamber containing 16 wells was used to improve visibility for larger number of samples at one time. Control cases were 10, and its derived either from normal tissue samples of the same patients confirmed by histopathological examination or from

other cases of benign breast conditions because of limitations of getting normal tissue from normal individuals.

2.7. Statistical analysis:

To compare the sensitivity and the specificity between the various tests the corrected T- test was used. While the overall agreements were the proportional similarity (in both positive and negative) result for every two tests were to be compared (Showman, 1986). The analysis was stratified according to estrogen and lipid profile which were calculated as a measure of the association with risk of breast cancer.

Chapter Three

Results

And

Discussion

Chapter Three

Results and Discussion

3.1. Stratification of Risk factor

The results covered 25 cases of patients diagnosed as breast cancer (group 1) and 10 samples of controls (group 2), all of the women (patients and control) were above 40 years (their mean age 50 years) .The present study investigated a correlation between some etiological risk factors and incidence of breast cancer. Data of patients were distributed according to selected characteristics major risk factors for breast cancer while control samples subjected to some of these criteria except for affected side as they were apparently healthy women (Tables 3-1). The screened factors were: age, family history, blood groups and age at menarche. Other risk factors such as smoking and drinking were excluded based on our population habits and as Iraqi women are generally none smokers and do not drink alcohol. Instead, the study focused on previous risk factors as well as the relationship of blood groups because most studies ignore this factor and up to our knowledge there is no genetic study about breast cancer pointed to this kind of relationship.

3.1.1. Age:

Overwhelmingly, age is the most significant risk factor for breast cancer development. At least 78% of patients were in their 40 and50 years old or older, and the incidence rate was increasing most rapidly among post-menopausal women.

However, the incidence of breast cancer in women under the age of forty is also increasing. Thus, the results of the present study, came in agreement with a group of researchers both in USA and Australia who reported that mutant genotype in breast cancer women increased sharply after the age of 40 years old (Wu *et al*., 2002; Zimmerman, 2004).

Regarding group1 (25 cases of breast cancer), the results of studied group, presented in table (3-1), showing distribution of the cases according to age, the mean age of breast cancer women was 49 years, so that the distribution of patient was significantly occurred between 40-49 years age ($p \leq 0.05$). Regarding the group 2, 10 normally healthy women and their age distributed between 40-55 years old as shown in table (3-1).

Table (3-1): Distribution of Breast Cancer Women and Controls According to Age Groups.

Patients age	Cases		Mutant genotype	Normal genotype	Control	
	No.	%	No.	No.	No.	%
40-49	16	64	8	8	5	50
50-59	6	24	4	2	4	40
≥60	3	12	3	0	1	10
Total	25	100	15	10	10	100

Numerous results obtained by other studies done in our country focused on the age of women with breast cancer like the study of Ghanim in (2009) who found the mean age of breast cancer women was 42.2 with rang of (26-71) years and the study of Al azawee (2006), who found that the mean age was 50.4 years with range of 22-85 years. In other part of the world like in the United Kingdom, where the age standardized incidence and mortality for the breast cancer is the highest in the world and the disease is the single commonest cause of death among women aged 40-50 years, accounting for about a fifth of all deaths in this age group.

3.1.2. Family history

Many studies concluded that women with a family history of breast cancer in first or second degree relatives are at a higher risk of developing breast cancer. The risk was 1.5 to 3.0 times higher if a mother or sister has the disease. Having both mother and sister with breast cancer increased the risk of breast cancer up to six fold. If that relative had bilateral breast cancer or was diagnosed at an early age, the risk may be further increased (Parkin *et al.*, 1992; Kelsey *et al*., 1996).

This study included 25 patients, 18 patients (72%) with breast cancer and have family history and 7 (28%) of them had no family history and this result confirmed the other result reported by previous worker as mentioned above but conflict with other Iraqi study by Aziz (2006) who reported that there is no significant difference breast cancer. The present observation indicated that there were 12 from 18 breast cancer patients with a family history of breast cancer and

Three of seven patients with no family history of breast cancer have positive relationship with BRCA1 and BRCA2 mutation.

Table (3-2) Association between Family History and Breast Cancer Women Cases

groups	family history	Total No	Normal genotype	Genetic mutation
patients	Yes	18	6	12
	None	7	4	3
Total		25	10	15
control	Yes	4	4	0
	None	6	6	0
Total		**10**	**10**	**0**

Women who have a family history of breast cancer are at a higher risk for it than those who lack such a history also women who have an especially strong family history (e.g., two or more first-degree relatives [a mother, daughter, or sister] with breast cancer, particularly before menopause) have a greater than 50 percent chance of developing breast cancer. This represents an approximately five- to 10-fold increase in a woman's baseline risk of developing breast cancer (Gail *et al*., 2007) and one of the main factors responsible for this elevated risk has been identified an inherited genetic mutation in one of two genes, BRCA1 and BRCA2, are particularly at risk of developing breast cancer (Nusbaum, 2007). Finally, Evan *et al* (2002) suggested that women with BRCA mutations

has an 80% lifetime risk, and women with no mutation but a strong family history have about a 40% lifetime risk.

3.1.3. Blood Group:

Polymorphism of blood groups in human populations has been investigated in correlation with the appearance of certain types of tumors, the degree of malignancy, the response to therapy and survival (Le Pendu *et al*., 2001). This factor was not included as a risk factor in other studies but many clinical investigation referred to some breast cancer patients of certain blood groups had recurrence after successful therapy.

The present study tested 25 blood samples of patients and the number of patient with blood groups A, B, AB and O were 16, 2, 1 and 6, respectively. The numbers of Control individuals A, B, AB and O were 6, 1, 1, and 2, respectively.

Table (3-3) Distribution Breast Cancer Women According to the Blood Group.

Blood groups	Patients		Controls	
	N0 of blood samples	Percentage %	N0 of blood samples	Percentage %
A	16	53.3	2	29
B	2	13.3	1	10
AB	1	6.7	1	10
O	6	26.7	6	60
Total	25	100	10	100

According to other results, the protective effect of blood group O on cancer development can be ascribed to increased apoptosis resistance of epithelial cells presenting A antigen (Marionneau *et al*., 2002). The higher prevalence of blood group A in this study was agreed with observation of (Tryggvadottir *et al*.,2000), who reported 2 fold higher prevalence of blood group A in familial breast cancer compared with the sporadic cases so the blood group A consider one of the most significant risk factors for a rapidly progressing breast cancer . Women with blood type A have been observed to have poor outcomes once they are diagnosed with breast cancer. In complete opposition to this blood type A tendencies, It was found that blood type O infers a slight degree of resistance against breast cancer, and even among patients, blood type O showed a significantly lower risk of death (Dadamo *et al*., 2001). Also many risk factors are associated with the development of breast cancer, it is seldom mentioned that blood type has an influence on susceptibility and outcomes.

The present study showed high incidences of breast cancer were observed in patient's samples with blood group A and O when compared with the control samples. Significant ($p \leq 0.05$) differences were noted. So A and O blood group may be considered as risk factors for breast cancer in the present study. The increased risk for developing breast cancers in individuals with blood group A may be attributable to expression of an A-like antigen (also called the Forssmann or Tn antigen). So-called incompatible A expression was detected in breast cancer. Cancer cells of this tumor are capable of A antigen expression even in individuals with blood group B or O. Thus, antibodies to A can attack precancerous and cancerous cells expressing this antigen. Individuals with blood groups A lack antibodies to A and so are more likely to develop these carcinomas (Hakomori, 1999).

In fact, some researchers have even gone so far as to say that "blood groups were shown to possess a predictive value independent of other known prognostic factors" when discussing breast cancer (Holdsworth *et al*., 1995). Research indicates that blood type A women are over-represented among breast cancer patients, and that this trend occurs even among women thought to be at low risk for cancer (D'adamo, 2001). The present result came in accordance with all the above studies and in contrast to observation of higher prevalence of group B among breast cancer women (George *et al*., 1996).

3.1.4. Age at menarche:

Epidemiological studies have consistently found that age at menarche and age at menopause are associated with breast cancer risk (Bernstein *et al*., 1993). It is well established that an early age at menarche is a risk factor of breast cancer (clavel-chapelon *et al*., 2002). The current study has indicated that 16 cases (64%) of samples have had a normal age at menarche (after age 12-14 years old); other 9 cases (36%) of samples have had an early menarche (before age 12-14 years old). The results showed no significant ($p \geq 0.05$) relation of that character to the risk of breast cancer.

The effects of age at menarche are linked to greater exposure to estrogen, which are promoters of breast cancer (kuller *et al*., 1995).Women with an early age at menarche have long term increases in serum estradiol and lower serum sex hormone binding globulin (SHBG) concentration than women with a late age at menarche. Most recent studies found no association between breast cancer risk and age at menarche (Barnett *et al*., 2008), while age at menarche has previously been inconsistently associated with survival and results of some studies reported

an association between early age at menarche and reduced survival in breast cancer patients and women treated with modified radical mastectomy have a significantly poorer survival (Caleffi *et al*., 1998).

Table (3-4) Distribution of Breast Cancer Women According to Age at Menarche

Groups patient	No of cases	After (12-14) years	Before (12-14) years
50 >	16	10	6
50 ≤	9	6	3
Total	25	16	9
control			
50>	5	0	5
50≤	5	0	5
Total	**10**	**0**	**10**

3.1.5. Estrogen:

Estrogen has an essential role in development and maintenance of female sexual characters. It play crucial role in pathogenesis and progression of breast cancer. The biological effects of estrogen such as growth stimulation and differentiation of normal mammary tissue is mediated primarily through high affinity binding of estrogen receptors (Surekha *et al*., 2007).

Several factors are related to reproduction appear to predispose women to breast cancer. For example, women with early onset of menarche (menstruation begins before age of 12 years) or late menopause (menopause occurs after 55 years) have an increased risk of developing breast cancer (Hulka and stark, 1995).

The present study showed the elevated levels of estrogen are observed in 7 cases (70%) from 10 studied cases and 6 patients of them were premenopause women, remaining one patient was postmenopausal women .The other three cases (30%) have had a normal plasma estrogen (Table 3-5) .This indicates a significant association between breast cancer risk and elevated estrogen levels, it was clear that there is a relationship between high estrogen exposure and high breast cancer risk during the years when women had functional ovaries also Ghanim *et al* (2009) found that elevated levels of estrogen are observed in 19 cases (63%) from the studied cases. This indicates a significant association between breast cancer risk and elevated estrogen levels. Estrogen did not appeared to cause the DNA mutations that trigger the development of human cancer but estrogen did stimulate cell proliferation. One or more breast cells may already had DNA mutation that increased the risk of developing cancer. Then estrogen will cause these cells to proliferate along with the normal breast cells. The result will be an increase in the total number of mutant cells. Any of these cells might later acquire the additional mutations that lead to the onset of cancer. In other words, estrogen-induced cell production leads to an increase in the total number of existing mutant cells. These cells were at increased risk of becoming cancerous. So the chances that cancer might actually develop were increased (Yager *et al* 1996).

The data of this study was support the hypothesis that higher serum estrogen concentrations are associated with a higher risk of breast cancer in postmenopausal women (Cauley *et al*., 1999**).** Breast cancer was approximately five times as likely to develop in the women with high estrogen values as in those with low values (Thomas *et al*., 1997). Japanese Americans had the highest breast cancer rates and the highest estrogen levels, which seems to underline the link. This is interesting, because breast cancer rates have been on the increase among Japanese women both in the U. S. A and Japan. It may be that lifestyle factors are impacting hormone levels, perhaps by changing age at menstruation or other hormonal events (Susan *et al*., 2006)**.**

Table (3-5) Distribution of Breast Women Cases According the Estrogen Level.

	Elevated	**N0 %**	**Normal**	**N0 %**	**Total**	**Mean ± S.E**	**Normal Values of Estrogen = 30-200 Pg/ml**
Cases	7	70	3	30	10	229.5±41.3	
controls	2	20	8	80	10	67.99+5.834	

3.1.6. Lipid Profiles:

Levels of circulating lipids and lipoproteins have also been associated with breast cancer risk, though published results have been inconsistent. Plasma lipids (i.e., total cholesterol [TC], high-density lipoprotein [HDL], and triglycerides [TG]) were analyzed for10 patients with invasive breast disease and 10 controls and that clarified Plasma TC ($p > 0.05$) was none significantly higher in patients compared with controls. There was no correlation found between total cholesterol level and breast cancer survival in all the age groups during present study. Overall total cholesterol level was not highly significant in breast cancer of women and control group (Ray et al., 2001). The increase in cholesterol levels of postmenopausal patients was only (2%). Some other researchers could not however establish any association between total serum cholesterol levels in women more than 40 and breast cancer risk (Gaard *et al*., 1994) while Abu-Bedair *et al*. (2000) who reported that the significantly increased level of TC in the breast cancer patients compared to the controls indicates that, there is an association between TC and breast cancer risk.

In the present study showed that the HDL level was not significant ($p>0.05$) in the breast cancer's patients these results were reinforced by Ray et al., (2001) who cleared that there was no significant effect of HDL on breast cancer patients. This result came in agreement with Ballard-Barbash (1994) who suggested that although, very weak or no association has also been reported.

On the other hand, there was no significant ($p>0.05$) change in serum triglyceride levels between the pre-postmenopausal patients and controls, a finding which was in agreement with the results of a study conducted by Gaard *et al*. (1994). However, Gooden *et al*. (1997) had reported elevated serum triglyceride levels in postmenopausal breast cancer patients also in a case-control study, Moyisch *et al*. (2000) reported that women with high serum triglyceride levels had an increased breast cancer risk. Disagreement results also observed by Hiten *et al*., (2008) who demonstrated that Serum triglyceride in postmenopausal cancer patients were higher than the control. Several studies had investigated the role of diet especially dietary fat, in the etiology of breast carcinoma, but its significance has remained controversial (Wu *et al*., 2000). Kolonel *et al*. (1983) suggested that elevated lipid levels precede the development of obesity and breast cancer and thus, might have an aetiological or predictive significance.

Table (3-6) Distribution of Breast Women Cases According the Lipid Profile.

	No.	Mean ± S.D of T.G	Mean ± S.D of T.C	Mean ± S.D of HDL
Patient	10	109.2±6.239	146.6±11.02	111.8±11.48
Control	10	98.2±5.646	95.5±11.343	100.1±4.89

3.2. Molecular genetic analysis:

The study included molecular genetic analysis of genomic DNA extracted from biopsy samples of 25 patients with breast cancer and ten as control.

3.2.1. Genomic DNA isolation from tissue samples:

In this study, the quantities of DNA obtained from biopsy of normal women and breast cancer patients were equal or less than 20 µg, and the purity of prepared DNA was 1.7- 1.9. The PCR technique does not required large quantities of DNA (Rafalski *et al*., 1997), but it requires highly purified DNA (Strauss *et al*., 2002). Hence, the genomic DNA obtained by DNA extraction kit was found to be suitable for the purpose of experimental work designed in this research.

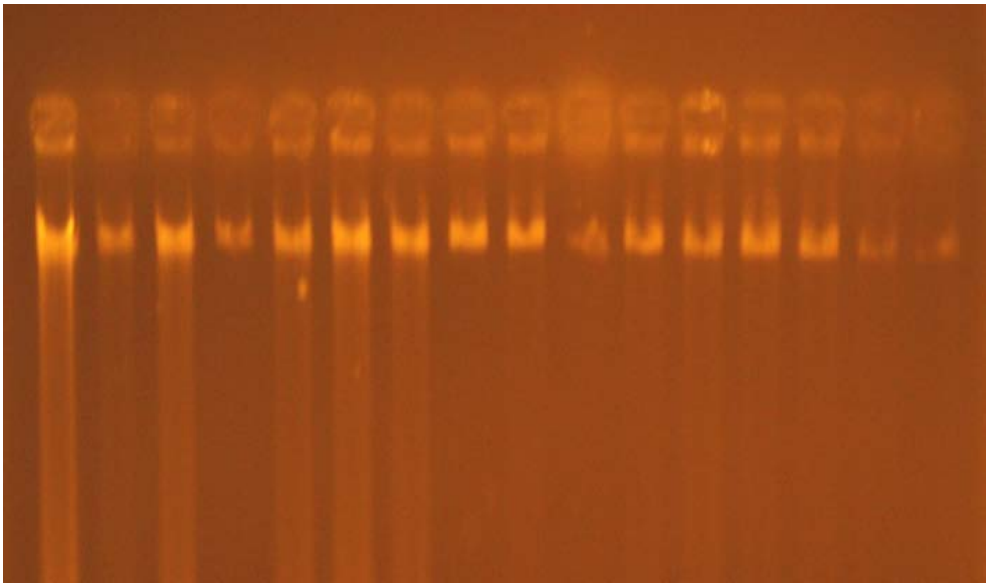

Figure (3-1): electrophoresis of DNA samples by 0.8% agarose gel (100V, 30 min).

The PCR-amplified exons 2 and 20 of the BRCA1 gene and exon 11 of the BRCA2 gene together with the adjacent regions of the boundary introns obtained from DNA of the patients with breast cancer were annealed with amplified control DNAs figure (3-1). The amplified DNA of patients that do not carry these mutations does not form heteroduplexes when annealed with amplified fragments of normal DNA; however, it forms heteroduplexes when annealed with amplified cloned DNA carrying the mutation (Mandelshtam*, et al*., 2001).

3.2.2. Genetic factors:

The frequency of BRCA mutation in the general population is unknown, making it difficult to calculate the increase in the risk of breast cancer in a carrier who does not have a strong family history of breast cancer. In breast cancer women, age-specific indicator refers to that breast cancer will develop in a carrier of mutation by a certain age. (Table 3-7) Presented that 8 of 16 women of breast cancer less than 50 ages have genetic mutation, 6 of them have BRCA1 whereas 2 have BRCA2 mutation while 7 of 9 women above 50 years have these mutation that clarified that the risk of mutation increased with age which lead to increase the breast cancer. Miki *et al.,* (1997) recorded that Breast cancer is occur approximately 20 percent by the age of 40 and 80 percent by the age after 50 years. Women with harmful BRCA1 or BRCA2 mutations often develop breast cancer after age 50 (Lynch, 2008), and Women carrying the BRCA1 mutation have an 85% risk of developing breast cancer in whereas Women with the BRCA2 gene mutation have an a 27% risk of developing breast cancer above 45 years of age (Liort , 2007).

Table (3-7) Association between Age and BRCA mutations.

No of patient	No of cases	No of mutation	BRCA1 185delAG	BRCA1 5382insC	BRCA2 6174delT	Normal genotype
≤50	16	8	3	3	2	8
≥50	9	7	1	5	1	2
total	25	15	4	8	3	10

All patients were analyzed for constitute BRCA mutation. The genomic DNA was used to detect the mutations by using 9 primers. The gremlin BRCA1 185del AG mutation was detected in 4 patients (16%), in this mutation the mutant and wild type amplicons showed the bands at 354bp and 335bp fig (3-2).

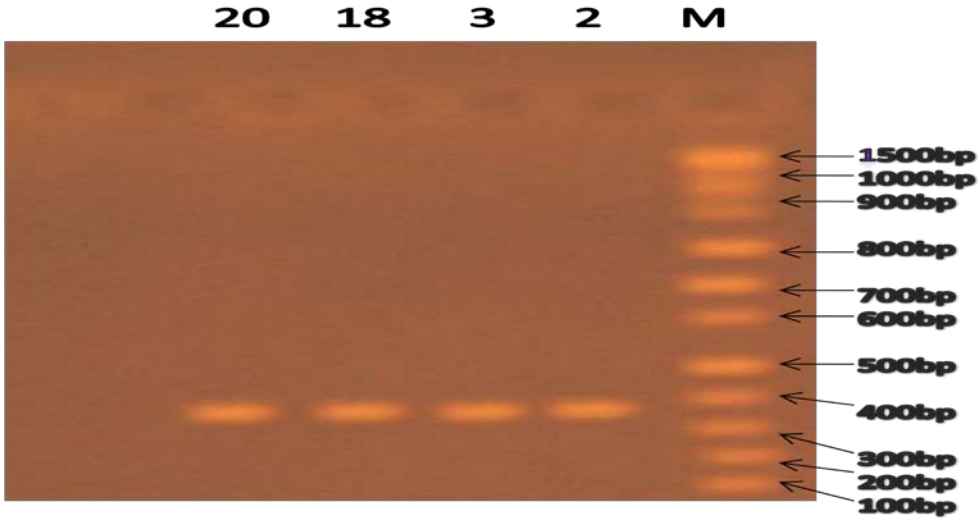

Figure (3-2):- Agarose gel electrophoresis (2%) showing BRCA1 185delAG detected by (100 V., 45 min) PCR Amplification, Lane **2, 3, 18, 20** showed mutant types. M: DNA marker (100 -1500bp).

whereas, the patients with BRCA1 5382insC appeared in 8 patients (32%) in this mutation the mutant and wild type amplicons showed band at 295bp and 271bp as show in the fig (3-3).

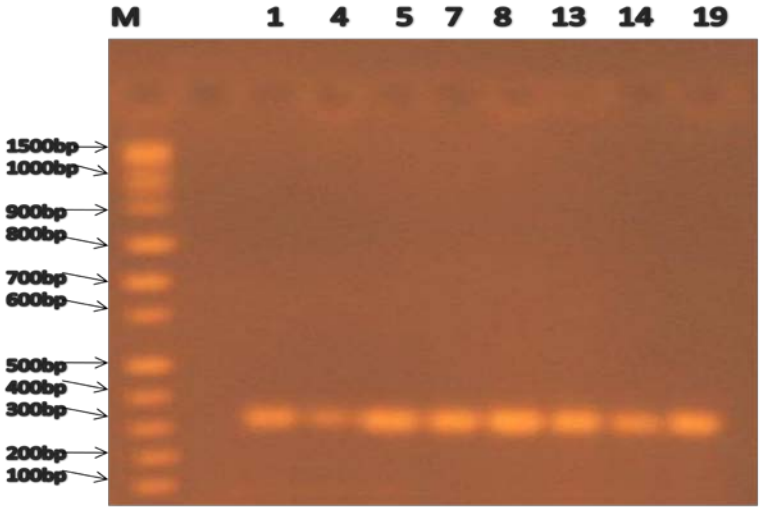

Figure (3-3):- Agarose gel electrophoresis (2%) showing BRCA1 5382insC detected by (100 V., 45 min) PCR Amplification, **Lane 1, 4,5,7,8,13,14,19** show mutant type, M: DNA marker (100 -1500bp).

In the present study it is clearly determined that BRCA1 5382insC was more frequency (32%) than BRCA1 185delAG (16%).This result was in agreement with (Struewing *et al* 1997) concluded that the breast cancer risk was highest for the 5382insC mutation in BRCA1 and Brose (2002) found comparable results, with a higher breast cancer risk in BRCA1 5382insC carriers. Others found that the penetrance of 185delAG and 5382insC were comparable, although the latte mutation was observed much more often than expected, suggesting a higher penetrance (Levy-Lahad *et al*., 2007).

The BRCA1 mutation involving insertion of a cytosine in BRCA1 (5382insC) probably originated from the Baltic region. It is the most common BRCA1 mutation worldwide, and is particularly important in Russia and Poland (Liede, 2002), also High frequency of BRCA1 5382insC was found in breast cancer families from western Russia (Gayther *et al*., 1998), so that confirmed the results of present study In Poland, a survey of families with breast or ovarian cancer reported that 5382insC represents 55.7% of the total BRCA1 mutations (Kittles *et al* 2003). 5382insC was the most frequently occurring BRCA1 mutation in studies in Greece (45%), the Czech Republic (37.3%), Hungary (28.6%), and Germany (21.7%) (Ladopoulou *et al*., 2002). The third mutation was BRCA2 6174delT, present in 3 patients (12%), BRCA2 6174delT showed two bands of mutant and wild type at 171bp and 151bp as fig (3-4).

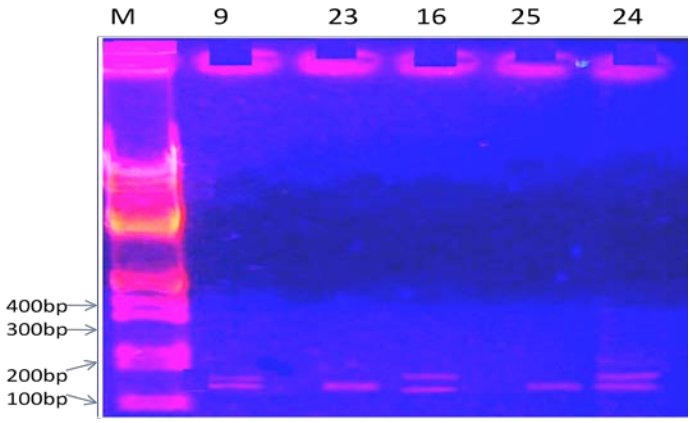

Figure (3-4):- Agarose gel electrophoresis (2%) was showing BRCA2 6174del T detected by (100 V., 45 min) PCR Amplification, lane 9, 16, 24 show mutant type, but 23, 25 show wield type. M: DNA marker (100 -1500bp).

The results of the present study agree with Levy-Lahad *et al*., (1997) who found the mutations 185del AG and 5382ins C in BRCA1were about 60% and 6174del T BRCA2 were about 30% of breast cancer incidence in Ashkenazi Jewish population. It was found that the penetrance of BRCA1 185del AG and 5382insC were found to be significantly higher than that of BRAC2 6174T and the frequency of BRCA1 mutation 48% was higher than BRCA2 (12%) and this study by Easton *et al*., (1994) found that the incidence of BRCA1 mutation was 87% and incidence of BRCA2 was 26% . In addition to Huusko *et al*., (1998) found that the frequencies of BRCA1 and BRCA2 gene mutations varied greatly in the large scale of breast cancer pedigrees of different countries and populations, which included 21% and 9% in Britain, 24% and 18% in France, 40% and 16% in Canada, and 39% and 25% in the USA, respectively. The remaining ten patients (40%) having normal genotype of BRCA1 & BRCA2 figure (3-11) and figure (3-12).

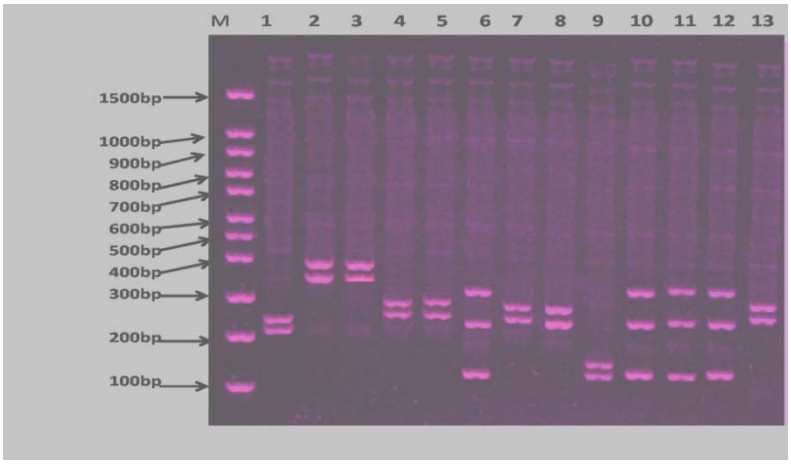

Figure (3-5):- Electrophoresis of PCR Products on Agarose gel 2% (100 volt / 45min). **Lane 1, 4, 5, 7, 8, 13:** 5382ins C BRCA1 (mutant type =295 bp and wiled type=271 bp), **Lane 2, 3:** 185del AG BRCA1 (mutant band =354 bp and wiled type=335 bp), **Lane 6, 10, 11, 12:** Negative control, **Lane 9:** 61742del T BRCA2 (mutant type =171bp bp and wiled type=151 bp) and **M:** DNA marker (100 -1500bp).

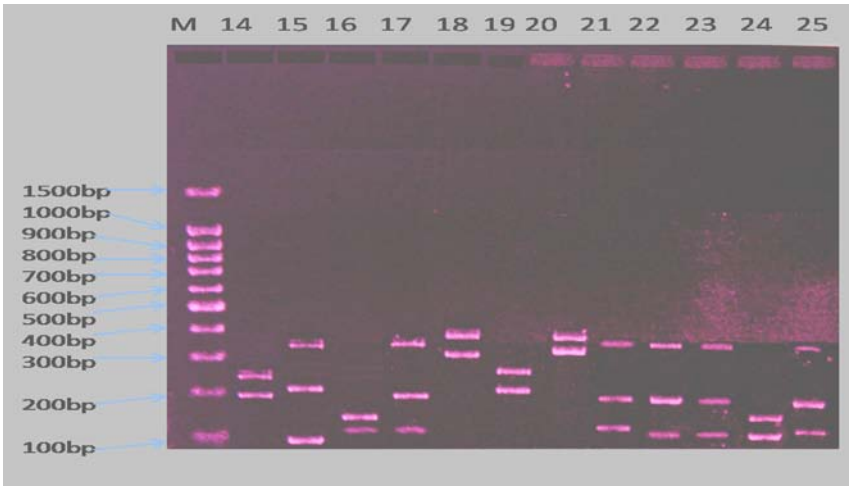

Figure (3-6) Electrophoresis of PCR Products on Agarose gel 2% (100 volt / 45min). **Lane 1 4, 19:** 5382ins C BRCA1 (mutant type =295 bp and wiled type=271 bp), **Lane 18, 22:** 185del AG BRCA1 (mutant band =354 bp and wiled type=335 bp), **Lane 15, 17, 20, 21, 23, 25:** Negative control, **Lane 16, 24:** 61742del T BRCA2 (mutant type =171bp bp and wiled type=151 bp) and **M:** DNA marker (100 -1500bp).

Finally it was cleared that BRCA1 and BRCA2 together account for approximately 80% of all hereditary breast cancer cases (Ford *et al*., 1998).

Chapter Four

Conclusions

And

Recommendations

Conclusions:-

1- The study demonstrated that the frequency of BRCA1 mutation (48%) was more than that of BRCA2 (12%) in women above 40 years.

2- No significant effect was found between lipids profiles (TG, TC, and HDL) and breast cancer patients.

3- A significant association was reported between elevated estrogen levels and breast cancer risk.

Recommendations:-

1- PCR specific primer amplification should be used as an important rapid tool for the early diagnosis of breast cancer in Iraqi population.

2- Using specific primers of other types of mutations of BRCA1 and BRCA2 and investigate the relationship between these mutations and breast cancer.

3-Further larger studies using large number of mutations of other genes are recommended to shed more light on the molecular diagnosis of breast carcinogenesis.

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Appendix

APPENDIX I

Department of Biotechnology/ AL-Nahrain University

(Breast Cancer Project)

1. Name of patient _____.

2. Place of birth _____

3. Address _____

4. Age ____

5. Blood group ____

6. Marital Status _____

7. No. of Children _____

8. Type of feeding: Breast Milk powder

9. Family History (Sister, Mother) _____

10. Age at Menarche _____

11 Age at Menopause _____

12 Oral contraceptive: Yes No

13. Date of Diagnosis _____

14. Affected side: Left Right Both

15. Date of Surgery: _____

16. Date of Sample _____

APPENDIX II

Breast cancer patients and their characteristics

No. of cases	Age of patients	Menstrual status	Age at menarche	Blood group
1	60	Menopause	>12	A
2	45	Distributed	≤12	A
3	40	Regular	≤12	B
4	50	menopause	≤12	O
5	45	menopause	≤12	A
6	53	menopause	≤12	A
7	57	Post menopause	>12	O
8	46	menopause	>12	A
9	55	Post menopause	≤12	B
10	45	Disturbed	≤12	O
11	47	Disturbed	>12	A
12	56	Post menopause	>12	A
13	49	Post menopause	>12	A
14	48	Disturbed	≤12	O
15	45	Regular	≤12	O
16	45	Disturbed	≤12	A
17	46	Disturbed	≤12	O
18	70	Post menopause	≤12	A
19	41	Regular	≤12	A
20	44	Disturbed	>12	AB
21	63	Post menopause	≤12	A
22	58	Post menopause	≤12	A
23	42	Regular	>12	A
24	40	Regular	>12	A
25	43	Regular	>12	A

APPENDIX III

Control Individual and their characteristics

Cases No.	Age	Menstrual status	Age at menarche	Blood group
1	43	Regular	≥12	O
2	51	menopause	<12	AB
3	50	menopause	≥12	O
4	42	Regular	<12	O
5	55	Post menopause	<12	A
6	48	Disturbed	≥12	O
7	53	menopause	≥12	O
8	57	Post menopause	<12	O
9	41	Regular	≥12	B
10	40	Regular	<12	A

الخلاصة

يعد سرطان الثدي السرطان الاكثر شيوعا الذي يصيب النساء في مختلف انحاء العالم . وقد تناولت الدراسات والبحوث مختلف العوامل المسببة لهذا المرض من اجل التوصل للتشخيص المبكر والعلاج الناجح له.

يعد سرطان الثدي السرطان الاكثر شيوعا الذي يصيب النساء في مختلف انحاء العالم .وقد تناولت الدراسات والبحوث مختلف العوامل المسببة لهذا المرض من اجل التوصل للتشخيص المبكر والعلاج الناجح ل ه. تناولت هذه الدراسة الحالية العلاقة بين التغايرات الوراثة للجينين **BRCA1** و **BRCA2** وعدد من العوامل الخطر ة المسببة لسرطان الثدي في النساء. واجريت على 25 مريضة بسرطان الثدي من مراجعي مستشفى الكاظمية التعليمي و 10 نساء طبيعيات ظاهريا تمثل مجموعة السيطرة جميع النساء (المريضات والسيطرة) كن باعمار فوق الاربعين سنة . كما تضمنت الدراسة العوامل آلاتية : العمر وصنف الدم وسن البلوغ وحاله انتظام الدوره الشهرية وتاريخ العائلة بالنسبة الى سرطان الثدي أو اي نوع اخر من الاورام و هرمون الاستروجين.

اخذت عينات دم من 10 مريضات بسرطان الثدي و 10من حالات السيطر ة و تم قياس مستوى الاستروجين فضلا عن قياس المستويات الانواع المختلفة للدهون في الدم وايجاد العلاقة بين هذ ه العوامل وخطورة الإصابة بسرطان الثدي. اوضحت النتائج وجود علاقة معنوية عالية بين مستوى الاستروجين في الدم والاصابه بسرطان الثدي، اذ وجد ان مستوى الاستروجين كان مرتفعا في 70% من مريضات المصابات بسرطان الثدي . وتعكس هذه النتائج العلاقة ا لايجابية لهرمون الاستروجين وخطور ة الإصابة بسرطان الثدي، بينما لم تظهر اي علاقة معنوية بين مستويات الانواع المختلفة للدهون (الجليسيريد الثلاثي (**TG**)، والكولسترول الكلي (**TC**) والدهن العالي الكثافه (**HDL**)) وخطوره الاصابه بسرطان الثدي . عند استعمال العده المستخدمه من شركه بروميكا تم استخلاص 50 مايكروليتر من DNA لكل 200-100مايكروغرام من النسيج المأخوذ من عينات نسيجية لنساء المصابات بالسرطان وبنقاوة تراوح من (1,7-1,9). استخدمت هذه الكميات من DNA لتضخيم جيني **BRCA1** و **BRCA2** بواسطة تفاعل البلمرة المتسلسل (**PCR**) وتم تحديد الطفرة. وأجريت هذه العملية للكشف عن وجود الطفر ة في ج ينيي **BRCA1**و **BRCA2** ان وجدت.

اظهر التحليل الوراثي ان نسبة الحذف في مريضات سرطان الثدي كانت 16% (4 مريضات) للطفرة **185 del AG** الموجودة في جين **BRCA1**، اما نسبة الإضافة للطفرة **5382 ins C** الموجودة في جين **BRCA1** للمريضات المصابات بسرطان الثدي 32 % (8 حالات)، بينما وجدت الطفرة الاخيرة **6174 T** في الجين **BRCA2** موجودة في ثلاث حالات بنسبة (12%). واستنتج من هذه الدراسة ان نسبة حدوث الطفرة في جين **BRCA1** 48% هي اعلى من نسبة حدوث الطفرة في جين **BRCA2** 12% في العيّنة من النساء العراقيات المصابات بسرطان الثدي.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

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رسالة مقدمة إلى
كلية العلوم – جامعة النهرين
وهي جزء من متطلبات نيل درجة ماجستير علوم
في التقانة الاحيائية

من قبل
ساره صالح حسن سدخان
بكالوريوس تقانة إحيائية، كلية العلوم، 2006
جامعة النهرين

بأشراف

أ.د.امنه نعمه الثويني أ.د.وليد حميد يوسف

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