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Genetic and Biochemical Study for Breast Cancer Women below 40 Years in Iraq

A Thesis

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بسْــمِ اللهِ الرَّحْمَنِ الرَّحِيْــمِ

وإذا مرضتُ فهوَ يَشْفين ☆ والذي
يميتني ثم يحين ☆

صَدَقَ اللهُ العَظِيمُ

سورة الشعراء الآية 72

الإهداء

إلى حِصني المنيع وبوابة العز، إلى من علمني الحياة من الألف إلى الياء ويبقى معلمي الأول مهما بلغت من العلم درجة سيبقى الفضل لك...

والدي الحبيب

إلى جنتي ورضا الله عليَّ إلى مَنْ صاغَ دمعَهُ قلائداً أتوسمها لتملأني أصراراً وقوة إلى مَنْ أهدى العمرَ كلّهِ وأشعلهُ شمعةً ليُنيرَ دربي فرحاً...

والدتي الغالية

إلى عمري الجميل، وحضني الدافيْ إلى من صارت فيَّ روحاً وقلباً وكيان، إلى مُحتلي الرقيق الذي قاسمني الروح والقلب قبل الوفاء ،إلى حلمي ورفيقة مشواري الطويل صدقَ من قال" رُبَّ أخٍ لم تلدهُ لك أُمك" وأنتِ نِعمَ الأخت والرفيقة والسند....

أختي الوحيدة"سارة"

إلى مَنْ كانوا سندي وقوة أعتمادي إلى مَنْ فخِرتُ بحمل دمهم بينَ عروقي وانتسابي بهم...

أخويَ"سيف-رامي"

إلى شمسٍ أشرقتْ علينا وفينا وملأتنا حناناً وأرتباط، إلى مَنْ كانتْ الرفيقُ الطيبُ الحنون...

زوجة أخي"شمس"

إلى كلِّ مريضةٍ ألهمتني العزمَ مِن معاناتِها وصبرِها على ما ابتلاها الله عزَّ وجلّ، إلى كل عائلة علّقت آمالها صوبي وهي ترجو الى مَنْ يُنقِذ عزيزَها...

إليكم جميعاً أُهدي فضلَ الله عليَّ

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Hamsa

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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List of Abbreviation

No.	Abbreviation	Meaning
1	BRCA1	Breast cancer gene 1
2	BRCA2	Breast cancer gene 2
3	DNA	Deoxyribonucleic acid
4	MOH	Ministry of health
5	W.H.O.	World Health Organization
6	DCIS	Ductal Carcinoma *In Situ*
7	LCIS	Lobular Carcinoma *In Situ*
8	LOH	Loss Of Heterozygosity
9	HDL	High-Density Lipoprotein
10	TC	Total Cholesterol
11	TG	Triglycerides
12	LDL	Low- Density Lipoprotein
13	VLDL	Very Low -Density Lipoprotein
14	GST	Glutathione *S*-Transferase Gene
15	HER-2	Human Epithelial Receptor 2
16	ssDNA	Single strand Deoxyribonucleic acid
17	PCR	Polymerase chain reaction
18	BRCA2 6174 del T	Deletion of base T in the gene BRCA2 at position 6174
19	BRCA1185del AG	Deletion of base pair AG in the gene BRCA1 at position 185
20	BRCA15382insC	Insertion of base pair C in the gene BRCA1 at position5382

Chapter One

Introduction

And

Literature Review

1.1. Introduction

Cancer is a general term that refers to cells that grow and multiply out of control and possibly spread to other parts of the body. Cancer can cause harm in different ways (Struewing *et al*., 2007). Each cancer cells take nutrition and space away from normal cells. A lump of cancer cells, called a tumor, can invade or destroy normal tissue also they can spread to other parts of the body; this is called metastasis (Altman and Sarg, 2000). One of the most dangerous types of cancer is breast cancer. However, breast cancer defined as cells in the breast divide and grow uncontrolled, these growth cells don't die at the proper rate and cell growth goes unchecked and cancer can develop (Osteen, 2001). In fact breast cancer is result of genetic alterations (mutations) in one of genes that may be included in breast carcinoma. These alterations may be inherited from family that carrying the same mutation. A few genes are responsible for an inherited predisposition to breast cancers. Two of these genes are called BRCA1 and BRCA2 (BReast CAncer 1 and 2). The majority of families with inherited predisposition to breast cancer have inherited alterations in these two genes (Kathryn *et al*., 2008).

There are three alterations in the BRCA1 and BRCA2 genes that are more common in individuals of different societies: Two alterations in the BRCA1 gene (185delAG and 5382insC) and one alteration in the BRCA2 gene (6174delT) that represent the vast majority of BRCA alterations families (Tang *et al*., 2001). Therefore, the recent developments in molecular genetics, including polymerase chain reaction (PCR) have 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 (Bar shack *et al*., 2000). Ultimately, the hope for these technologies is that they will improve our 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. PCR-based approaches might enable rapid and definitive testing for these genetic alterations (Thor *et al*., 2001).

Other factors shared in happening of breast cancer, one of these notable factors is the estrogen (the sex hormone) that stimulates the expression of genes encoding for growth factors and lead to increase proliferation of breast tissue and breast cancer cells. So the cell proliferation is essential for carcinogenesis because cell division increases the risk of errors during DNA replication, which if not corrected, can lead to breast cancer (Hoffman *et al*., 2000). In addition to these important factors lipid profiles also proved significant role in causing breast cancer in recent studies (Barbara, 2009). Lipid profiles be high with high body mass as well as the high body mass has strong relationship with breast cancer finally this relation consider as additional determinative factors for incidence of breast cancer (Michelle *et al*., 2003).

In the United States, there has been a sharp increase in the incidence of breast carcinoma, (Simon *et al*., 2008). According to cancer registry section (Iraqi cancer board) Baghdad / MOH, breast carcinoma is the most common malignant tumor in Iraqi women and it comprise (31.3%) of all female malignant cases in the United States, each year about 100,000 new cases are diagnosed and about 30,000 patients die from the disease.

Aims of study:-

In Iraq, breast cancer is common. Few molecular studies are available for assessing breast cancer. Therefore, we have two major aims in the current study:

First: Investigate the presence of 185del AG, 5382ins C and 6174del T in BRCA1 and BRCA2 genes in Iraqi women below 40 years.

Second: Investigate the relationship of developing breast cancer with estrogen hormone level and lipid profile.

1.2. Literature review

1.2.1. Breast Anatomy:-

The breast lies between the second and sixth ribs, from the sternal edge to the edge of the axilla, and against the pectoral muscle on the chest wall. Breast tissue also projects into the axilla as the tail of Spence. For clinical purposes, the breast is divided into four quadrants: upper inner, upper outer, lower inner and lower outer quadrants. Also it is composed of 15-20 lobes that radiate from the nipple. Each lobe is surrounded by fat and fibrous connective tissue and is divided into many lobules. The lobule (sometimes called the ductal-lobular unit) is the basic structural unit of the breast and is lined by epithelial cells. Each lobule is subdivided into 10 to 100 alveoli, the milk producing units of the breast. Milk flows from the alveoli of the lobules into the ducts. The ducts gradually coalesce into 10 to 15 major ducts; each lobe containing one major duct terminating in the nipple as shown in figure (1- 1) (American Cancer Society, 2006).

As well as the Breast contains blood and lymphatic vessels. Most lymphatic vessels within the breast lead to axillary lymph nodes, some also connect to supra- or infraclavicular nodes, and internal mammary nodes. They may enter lymphatic vessels and spread to lymph nodes. Beneath the tissues of the breast lie the muscles of the chest wall and between the two is the fascia (a layer of connective tissue). Two layers of suspensor ligaments (Cooper's ligaments) link the breast to the fascia, providing support. As these ligaments stretch with age or weight gain, the breast loses some of its firmness (Cress man *et al*., 1999).

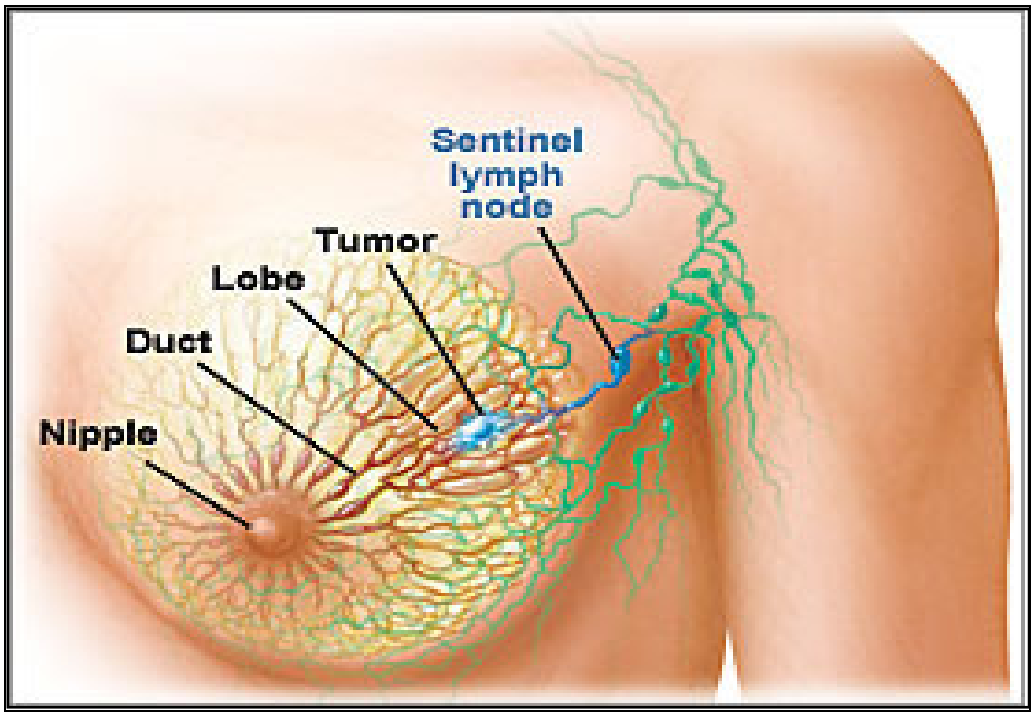


Figure (1 – 1) primary anatomical features of the breast indicating the ducts and lobules (Brandy, 2004).

1.2.2. Breast cancer:-

Breast tumor occurs when cells in the breast begin to grow out of control and then invade nearby tissues or spread throughout the body (Armstrong *et al.,* 2005). Large collections of this out of control tissue are called tumors (Cooper, 2000). A tumor may be either benign or malignant. A benign tumor remains confined to its original location, invading neither surrounding normal tissue nor spreading to distant body sites. A malignant tumor, however, is capable of both invading surrounding normal tissue and spreading throughout the body via the circulatory or lymphatic systems (metastasis). Only malignant breast tumors are properly referred to as cancer. Theoretically, any of the types of tissue in the breast can form a cancer, but usually it comes from either the ducts or the glands (Rhodes, 2002). Statistical data has shown that breast cancer constitutes 33.12 percent of the registered female cancer in Iraqi (Iraqi

Cancer Registry, 2002). The same observation has been noted in many other developing countries lying within the Eastern Mediterranean region (Alwan, 1995). In those areas reported rates of 31 percent, 30 percent and 26 percent have been documented in hospital based cancer registries of Egypt, Lebanon and Jordan respectively (Omar and Contesso, 1988). The annual incidence of breast cancer in the United states increases dramatically with age (5 per 100 population at 25 years of age, rising to 15 per 100 at 50 years of age and to more than 20 per 100 at 75 years of age). Moreover, the incidence of male breast cancer is about 2.5 per 100 populations (Casciato and Lowitz, 2000).

1.2.3. Symptoms: -

When breast cancer first develops, there may be no symptoms at all. But as the cancer grows, it can cause the following changes:

1. A lump or thickening in or near the breast or in the underarm area or in the neck.
2. A change in the size or shape of the breast.
3. Nipple discharge or tenderness, or the nipple pulled back (inverted) into the breast (Pam, 2007).
4. Ridges or pitting of the breast skin (like the skin of an orange).
5. A change in the way the skin of the breast, areola, or nipple looks or feels (for example, warm, swollen, red, or scaly) (World Health Organization (WHO), 2003).

1.2.4. Epidemiology:-

Today, breast cancer, like other forms of cancer, is considered to be the final outcome of multiple environmental and hereditary factors. Some of these factors include:

1. Lesions of DNA such as genetic mutations. Mutations that can lead to breast cancer have been experimentally linked to estrogen exposure (Goldgar *et al*., 2004). Beyond the contribution of estrogen; research has implicated viral oncogenesis and the contribution of ionizing radiation in causing genetic mutation.
2. Failure of immune surveillance, a theory in which the immune system removes malignant cells throughout one's life (Linnea *et al*., 2001).
3. Abnormal growth factor signaling in the interaction between stromal cells and epithelial cells can facilitate malignant cell growth. For example, tumors can induce blood vessel growth (angiogenesis) by secreting various growth factors further facilitating cancer growth.
4. Inherited defects in DNA repair genes, such asBRCA1, BRCA2 and P53 (Hedau *et al.,* 2004).

1.2.5. Molecular pathology of breast cancer:

The multistep nature of carcinogenesis in general, as well as in breast carcinoma has been widely accepted. Similarly to other solid tumors, the development of breast cancer is through to be initiated after multiple successive changes in the genome of the cell in the “target” tissue (Bieche and Liderean, 1995). Early studies of the age-dependence of cancer suggested that on average 6-7 successive somatic mutations are

needed to convert a normal cell into invasive carcinoma cell (Ruan, 1993). Clinically, the genomic changes are manifested as pathologically aggressive growth, invasion and metastatic behavior of proto-oncogene mainly through amplification, and coordinated inactivation of tumor suppressor genes (Beckman *et al.,* 1997). However, in sporadic cancer, when near-normal breast cells evolve to malignancy, the genetic abnormalities developed are so diverse that it has been suggested that no two tumors or tumor cells in any one tumor are likely to be genetically identical (Lengauer *et.al.,* 1998). Many of the somatic genetic changes seen in sporadic cancers are present more frequently in *BRCA1* and *BRCA2* epigenetically mutated cells, confirming the nature of these breast cancer susceptibility genes as DNA stability conserving genes (Ingvarsson, 1999). Epigenetic lesions have been shown to drive genetic lesions in cancer (Esteller, 2002). When loss of heterozygosity (LOH) and methylation analysis are used, this may also be seen in the more malignant nature of the hereditary breast cancers as a whole. The development of breast cancer is known to involve many types of activated or inactivated genes in order to promote malignancy. Studies suggesting that both the structural and numerical aberration tend to be more complex in more malignant and aggressive tumor than in intraductal or less aggressive types of invasive carcinomas (Aubele *et. al*., 2000). There are various chromosomal regions which are frequently amplified, but no specific oncogene during this amplification has yet been identified (cuny *et al*., 2000).

1.2.6. Staging of breast cancer:

Cancer staging systems describe how far cancer has spread anatomically and attempt to put patients with similar prognosis and treatment in the same staging group. The staging system normally used in breast cancer is

called TNM, which stands for 'tumor, node, and metastasis. So TNM staging takes into account the size of the tumor, whether the lymph nodes are affected, and whether cancer has spread to other parts of the body (metastasis) (Zubair *et al*., 2009).

❖ **The T stages (tumor)**

➢ **TX-** Means that the tumor size cannot be assessed

➢ **T1 -** The tumor is no more than 2 centimeters (cm) across.

➢ **T1 is further divided into 4 groups:**

- **T1mic** - Under a microscope the cancer cells can be seen to spread less than 0.1cm into surrounding tissue (micro invasion)
- **T1a -** The tumor is more than 0.1 cm but not more than 0.5 cm
- **T1b -** The tumor is more than 0.5 cm but not more than 1 cm
- **T1c -** The tumor is more than 1 cm but not more than 2 cm

➢ **T2 -** The tumor is more than 2 centimeters, but no more than 5 centimeters across.

➢ **T3 -** The tumor is bigger than 5 centimeters across.

➢ **T4 is divided into 4 groups:**

- **T4a** - The tumor has spread into the chest wall.
- **T4b -** The tumor has spread into the skin.
- **T4c -** The tumor is fixed to both the skin and the chest wall.
- **T4d -** Inflammatory carcinoma - this is a cancer in which the overlying skin is red, swollen and painful to the touch (Jemal *et al.,* 2006).

❖ **The N stages (nodes)**

➢ **NX**- means that the lymph nodes cannot be assessed (for example, if they were previously removed)

- **N0** - No cancer cells found in any nearby nodes
- **N1** - Cancer cells are in nodes in the armpit but the nodes are not stuck to surrounding tissues
- **N2 is divided into 2 groups:**

- **N2a** - there are cancer cells in the lymph nodes in the armpit, which are stuck to each other and to other structures
- **N2b** - there are cancer cells in the lymph nodes behind the breast bone (the internal mammary nodes). These have either been seen on a scan or felt by the doctor. There is no evidence of cancer in lymph nodes in the armpit

- **N3 is divided into 3 groups:**

- **N3a** - there are cancer cells in lymph nodes below the collarbone
- **N3b -** there are cancer cells in lymph nodes in the armpit and under the breast bone
- **N3c -** there are cancer cells in lymph nodes above the collarbone (Goldhirsch *et al*., 2003).

- **The M stages (metastases)**
- **M0 -** No sign of cancer spread.
- **M1 -** Cancer has spread to another part of the body, apart from the breast and lymph nodes under the arm (Steve, 2009).

Tumor Grading

Tumor grade refers to a measure of how abnormal cells from your tumor appear under the microscope. This can refer to the appearance of the cells or to the percentage that appear to be dividing. The higher the grade, the more aggressive and fast

growing the cancer. Tumors are typically classified from least to most aggressive as grade I through IV (Zubair *et al*., 2009).

The grade is much more important for some kinds of cancers than for others. For most kinds, it is a somewhat secondary factor, but for a few kinds of cancers, notably certain brain tumors, prostate cancer, and lymphomas, it is extremely important. Again your doctor will know how your tumor was graded and how important it is to your type of cancer. The grading will also be found on the pathology report from your biopsy or surgery. For information on understanding pathology reports (White *et al*., 2008).

1.2.7. Risk Factors:-

1. Age:

Breast cancer risk is strongly related to age and it is the most commonly diagnosed cancer in women under 35 years. Among women aged 35-39 years around 1,500 cases of breast cancer are diagnosed each year. Breast cancer incidence rates generally increase with age, with the greatest rate of increase prior to the menopause, supporting a link with hormonal status.

Diagnosing breast cancer in younger women (under 40 years old) is more difficult because their breast tissue is generally denser than the breast tissue in older women. By the time a lump in a younger woman's breast can be felt, the cancer often is advanced. In addition, breast cancer in younger women may be aggressive and less likely to respond to treatment. Women who are diagnosed with breast cancer at a younger age are more likely to have a mutated (altered) BRCA1 or BRCA2 gene (Levi *et al*., 2007).

The 10-year overall survival probability of a 30-year old patient (85%) was equal to that of a 60-year old, indicating a considerably reduced life expectancy in young patients (Tavani *et al*., 2009). While the Barsky *et al*. (2001) suggested that carcinoma of the breast is extremely rare below the age of 20. this suggestion comes in agreement with Malone *et al*. (2000) who mentioned that breast cancer is occurs in small percentage in women below the age of 25 years, but incidence rates increased steadily from then, reaching over 300 per 100 000 of the population by the time women are 85 years old, the largest or greatest number of women are diagnosed between the age 35 to 75 years. Breast cancer in men is almost always seen beyond the age of 65 years.

2. Family history:

A family history of breast cancer means having one or more close blood relatives who have or have had a breast cancer. Close relatives are parents, siblings (first degree relatives) or aunts, uncles, nephews, nieces or grandparents (second degree relatives). When one of these family members have developed a breast cancer before menopause or has had cancer in both breasts, the risk of breast cancer will increase (Gregory *et al*., 2007). Family history of breast cancer does affect one's risk for developing the disease. Epidemiological studies have found that women with a family history of breast cancer in first or second degree relatives are at a higher risk (Vena *et al*., 2001). Therefore, the important features in a family history that seems to be study worthy are:

- Age at onset.
- Bilateral disease.
- Multiple cases in the family (particularly on one side).
- Other related early onset tumors.

• Number of unaffected subjects (large families are more informative) (Evans and Lalloo, 2002).

The risk is 1.5 to 3.0 times higher if a mother or sister has the disease (Parkin *et al.,* 2007). Having both mother and sister with breast cancer increases 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 (Wohlfahrt and Gammon, 2001). In small groups of families, the patterns of breast cancer seem to be consistent with the known patterns of genetic inheritance (Pharaoh *et al*., 1998). According to some studies, approximately 10% of breast cancers can be attributed to inherited mutations in breast cancer related genes. Most of these mutations occur in the BRCA1 and BRCA2 genes. Approximately 50% to 60% of women who inherit BRCA1 or BRCA2 gene mutations will develop breast cancer by the age above 30 years (Vena *et al*., 2001).

3. Menstrual and reproductive history:

Breast cancer risk increases with early menarche and late menopause, and is reduced by early first full-term pregnancy. (Pat hank, 2000)

The pattern of menses following menarche also influences breast cancer risk. The sooner regular menstruation is established, the greater the subsequent risk of breast cancer. Henderson *et al,* (1996) reported that women who have been actively menstruating for 35 or more years are thought to have twice the risk of developing breast cancer than women with 30 years or less of menstrual activity. The breast cancer linkage consortium data suggest that in families with four or more cases of early onset or bilateral breast cancer, the risk of an unaffected woman inheriting a mutation in a predisposing gene is close to 50%. Epidemiological studies have shown that approximately 80% of mutation

carriers in known predisposing genes (***BRCA1*** and ***BRCA2***) develop breast cancer in their lifetime (Gregory *et al*., 2007).

4. Sex:

Although most cases of breast carcinoma occur in female, in the United State only 1% of all breast carcinoma occur in male, but in Egypt and other countries the incidence rise to nearly 10% and which is known predispose factor of breast carcinoma (Garfunkel *et al*., 2000).

5. Smoking:

Epidemiological studies of smoking and breast cancer have been inconsistent. Some of these studies suggested some reduced risk of breast cancer among smokers, while others showed no association, 49 years or small increase in risk especially among heavy smokers. Cigarette smoking does not appear to influence risk except possibly in women who are slow acetylators of aromatic amines. Some studies have found an association between smoking and an increased risk of breast cancer, including exposure to second hand smoke (Johnson, 2005).

6. Ionizing radiation:

Ionizing radiation to the chest increases the risk of breast cancer. The magnitude of risk depends on the radiation dose, the time since exposure, and age. Only women irradiated before age 30, during breast development appear to be affected (Christopher *et al*., 2003)**.**

7. Lactation:

There is consisting evidence that lactation has either a protective effect against breast cancer, or a neutral one. Breast feeding reduces the number of ovulations proportionally to its duration and intensity, and maintains a

lower estrogen level than the level observed during the menstrual cycle (Michael *et al*., 2002).

8. Oral contraceptives:

A small increase in the risk of breast cancer has been noted in users of oral contraceptives. This risk, however, drops following the cessation of contraceptive use so that, at ten years post-use, there is no significant increase in the risk of developing breast cancer. Use of oral contraceptives at an older age has also been linked to an increase in the number of breast cancer cases diagnosed (Johnson, 2005).

9. Psychological factor

It has been postulated that women with certain personality traits are at greater risk of breast cancer and that severe stress may precipitate breast cancer. It is particularly difficult to disentangle psychological traits, prospective linkage of stressful "life events" such as bereavement to subsequent breast cancer incidence has failed to show that stress induces breast cancer (Alexander *et al*., 1999).

10. Alcohol:

Research has suggested that drinking alcohol may increase breast cancer risk, and the increased risk is tied to the amount of alcohol consumed. One proposed explanation for the relationship between alcohol and breast cancer is that alcohol consumption may increase the amount of circulating estrogen in the bodies of women who drink. Since

many studies show a relationship between alcohol consumption and increased breast cancer risk, it is important to consider this when deciding whether or not, and how much to drink (Swanson *et al*., 1997).

1.2.8. Biochemical effect of breast cancer:

1.2.8.1. Estrogen:-

The role of estrogens in affecting breast cancer risk at the age < 40 years has remained largely unknown. Several factors related to reproduction appear to predispose women to breast cancer. For example, women with early onset of menarche (menstruation begins at or before 12 years) or late menopause (menopause occurs after 55 years) have an increased risk of developing breast cancer (Hulka and Stark 2005).These findings suggest that the longer the exposure to ovarian estrogens, the higher the risk. This view was supported by the fact that surgically induced menopause before age 45 years and the resulting removal of ovarian estrogens markedly reduce breast cancer risk (Kreiger, 1999). In contrast to these adverse effects of estrogens on the breast, in certain circumstances, such as during pregnancy that occurs before age 20 years and during the prepubertal period and childhood, estrogens actually reduce breast cancer risk (Berkey and Hutter, 1999). The reduced risk could be achieved through estrogen-induced activation of certain tumor suppressor genes, including BRCA1 (Gregory *et al*., 2007). And p53 (Hurd *et al.,* 1997) that are critical in DNA damage repair and in maintaining genetic stability, thus reducing the likelihood that breast cancer will be initiated. The interaction between estrogens and tumor suppressors might be important during the early reproductive years, when the breast does not yet contain any malignancies. Once breast cancer initiation has taken place, estrogens might promote the growth of

transformed cells, leading to the development of detectable breast cancer (Spillman and Bowcock, 2006).

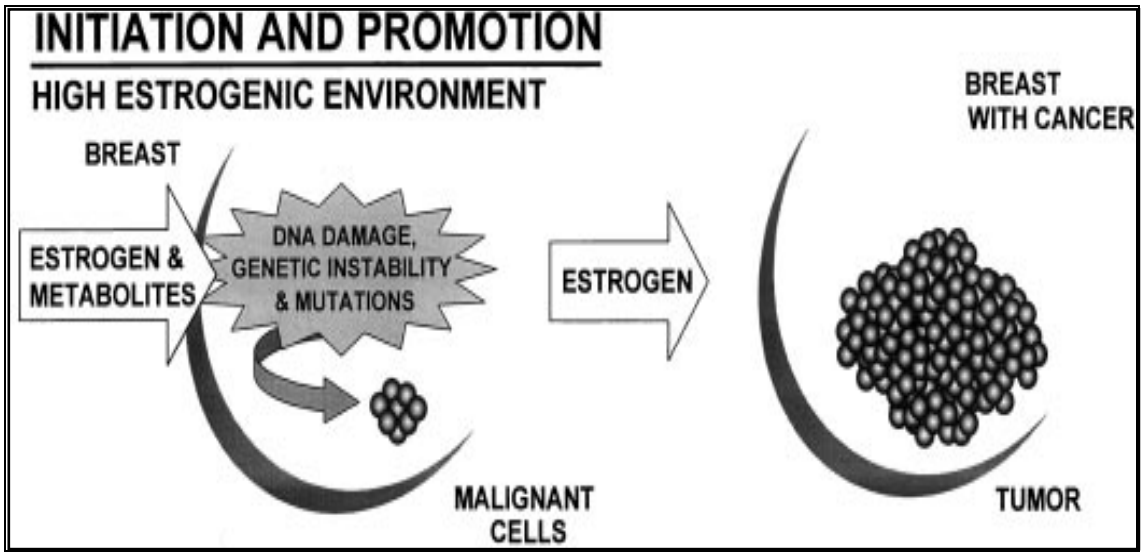


Figure (1-2) Estrogens may increase breast cancer risk by acting as a pre-initiator, an initiator (by inducing direct or indirect free radical mediated DNA damage), or a proliferators (stimulating the growth of existing malignant cells in post initiated breast) (Kreiger *et al*., 1999).

1.2.8.2. Lipid Profile:-

A lipid profile is a direct measure of three blood components: cholesterol, triglycerides, and high-density lipoproteins (HDLs). Cholesterol is a vital substance that body uses to produce such things as digestion-aiding material, hormones, and cell membranes. It is both produced by the body and absorbed from some of the foods eat. Cholesterol and triglycerides are transported in the blood by combinations of lipids and proteins called "lipoproteins". Too much cholesterol is harmful, since excess cholesterol can be deposited in blood vessel walls. These fat deposits can lead to atherosclerosis, or hardening

of the arteries, and cardiovascular disease, the number one killer in the United States. High levels of triglycerides are also associated with an increased risk of heart disease (Mahabir *et al*., 2006). HDLs, the so-called "good" or "healthy" cholesterol, are lipoproteins made mostly of protein and little cholesterol. HDLs can help to clear cholesterol deposits in blood vessels left by another blood component called low-density lipoproteins, or LDLs. LDLs and very-low-density lipoproteins (VLDLs) are the so-called "bad" cholesterols. Unlike HDLs, LDLs and VLDLs are high-cholesterol particles. Some medical conditions can raise or lower cholesterol and triglyceride levels. Factors such as age, sex, and genetics influence lipid profile (Furberg *et al*., 2004). Certain aspects of the lifestyle, including diet, level of physical activity, level of diabetes control, and smoking status, also affect lipid profile. Premenopausal (before 40 years) women who do not have diabetes tend to have better cholesterol levels than men, but this protective effect seems to be negated in women with diabetes. Both sexes show an increase in lipoprotein levels as they age (Mahabir *et al*., 2006).

1.2.9. Genes and breast cancer

1.2.9.1. BRCA1 gene (mutation):-

The discovery of breast cancer genes has led to an explosive growth in cancer screening for population at risk (Armstrong *et al*., 2005). Despite the genetic heterogeneity of breast cancer and the high prevalence of sporadic disease, several breast cancer susceptibility loci have been identified (Casciato and Lowitz, 2000). The first of these genes, named *BRCA1* (Hall *et al.,* 1990). The *BRCA1* gene greatly increases the life time risk of inheriting certain forms of breast and ovarian cancer (Petruceli *et al*., 2004). *BRCA1* carries will develop breast cancer in a percentage of 25 to 35 by age of 70 (Casciato and Lowitz, 2000). *BRCA*1

is a tumor suppressor gene, because the phenotype results from a loss of function. It was mapped in 1990 to chromosome 17q21 by genetic linkage analysis of large families that included many cases of early-onset breast carcinoma (Hall *et al.*, 2007). *BRCA*1 is a complex gene make up of more than 20 exons distributed over more than 100 kilo base (kb) of genomic DNA and encodes a 1863-amino acid protein, with two RING finger domains at its N- terminal part are thought to be involved in DNA- binding or in protein- protein interactions. *BRCA*1 shares a conserved region with 53bp1 (p53- binding protein) and radq (a yeast protein involved in the control of the DNA damage- induced cell cycle arrest), which has suggest that *BRCA1* is likely to function in the cell nucleus and may be involved in one or more cell cycle check points (Koonin *et al*., 1996). Mutations in the *BRCA1* gene are thought to account for about half of the families susceptible to early- onset breast cancer and for at least 80 percent of families with clustered breast and ovarian cancers (Couch and Weber, 1996).

Germline *BRCA1* mutation have been reported in more than 200 families from different geographic origins (Petruceli *et al*., 2004). The mutation which reported in these cases includes missense mutations, non sense mutations, deletions, insertions, or intronic mutations, although the majority results in the production of a truncated protein (Fitz Gerald *et al.*, 1996). Almost half (48 percent) of women in southern Sweden with early- onset breast cancer have some family history of breast or ovarian cancer and 9 percent of the cases are associated with a Germline mutation in *BRCA1*. Mutation carriers where more prevalent among young women. Women with at least one first- or second- degree relative with breast or ovarian cancer, and women with bilateral breast cancer (Loman *et al*., 2001).

1.2.9.2. BRCA2 gene (mutation):-

The observation that less than half the families with multiple cases of breast cancer showed linkage to *BRCA1* led to the proposal that there was at least an additional gene associated with breast cancer susceptibility. This result prompted another genomic linkage search and a second breast cancer susceptibility gene, named *BRCA2,* was located on chromosome 13q12 (Wooster *et al*., 2003) and subsequently cloned (Tavtigan *et al*., 2006). *BRCA2* is composed of 27 exons and encodes a protein of 3418 amino acids residues which does not appear to be significantly similar to other proteins (Rajan *et al.*, 1996). It is clear that BRCA2 is important components of the pathway that protects cells from the effects of DNA damage (Vaughn *et al*., 2004). Recent studies have been shown that *BRCA2* expression is coordinately regulated with *BRCA1* expression during proliferation and differentiation in mammary epithelial cells, suggesting that both genes may act in the same pathway (Rajan *et al.*, 1996). In addition, *BRCA2* forms complexes with both BRCA1 and Rad51, the human homology of the *Escherichia coli* gene *RecA*; this is essential to normal recombination and genome stability (Kufe *et al*., 2003). The majority of mutations identified thus far lead to protein truncation, and it is believed that cancer then develops when the second copy is lost. Therefore, it is thought that *BRCA2* behave like classic tumor suppressor gene, with the loss of one copy predisposing the carrier to the development of the characteristic cancers of this classic cancer syndrome. Between 35 percent and 50 percent of *BRCA2* carrier develop the disease (Phelan *et al.*, 1996).

1.2.9.3. Glutathione *S*-transferase Gene (GST):

GST is a gene of family with a critical function in the protection against electrophiles and the products of oxidative stress (Shen *et al.*, 2000). The GSTs are involved in the metabolism of many xenobiotics, including an array of environmental carcinogens and chemotherapeutic agents and endogenously derived reactive oxygen species. GSTs are widely distributed in nature and are found in essentially all eukaryotic species. The cytosolic glutathione transferases comprise four classes, α, μ, π and θ (A, M, P and T) three of which at least are represented in both normal and breast tumor tissue (Forrester, 2008).The four major families of GSTs, distinguished on the basis of primary structure, are designated as, μ, π, and θ and are encoded by the *GSTA*, *GSTM*, *GSTP*, and *GSTT* genes, respectively. Of these, class πand μ predominate in the breast (Rio *et al*., 1999). The glutathione S-transferase mu (GST-M1) and theta (GST-T1) are separated isoforms of glutathione transferase enzymes that participate in the metabolism of a wide range of chemicals, including possible carcinogens. The known substrates for the GST-M1 enzyme include reactive epoxide intermediates generated from the activation of polycyclic aromatic hydrocarbons by cytochrome P450 enzymes (Shen *et al.*, 2000). Exposure to polycyclic aromatic hydrocarbons from cigarette smoke and other sources is ubiquitous and has been shown to induce mammary tumors in animal models. GST-T1 enzyme substrates include chemicals with wide industrial use that can also cause mammary tumors in animals. However, the majority of studies reported no relation or even a possible inverse association of the null GST-M1 variant with breast cancer (da Fonte, 2002).

1.2.9.4. Human epithelial receptor 2 (HER-2)

(HER-2) a member of the class I growth factor-receptor tyrosine kinase family, the genetic abnormality is not inherited but acquired (Newman and Kuerer, 2005). The HER-2 (human epithelial receptor 2, also known as HER-2 gene is located on chromosome 17q and encodes a 185-kDa transmembrane tyrosine kinase growth factor receptor .The her2 is another gene found on the surface of cell that plays a key role in regulating cell growth. The over-expression of HER-2 increases cell growth and reproduction, often resulting in more aggressive tumor (Ren *et al*., 2002). When the gene of this protein is altered, extra HER2 receptors may be produced Her2 over-expression may not be very responsive to standard breast cancer treatments, including certain regimens of chemotherapy.The HER-2 gene is rarely amplified in benign breast disease, and its expression varies by histological subtype, as it is almost exclusively found in the primary breast cancers of ductal origin in contrast to those of lobular origin. The HER-2 gene is amplified and over expressed in 20%–30% of invasive breast cancer and, interestingly, in the majority of high-grade DCIS cases (Hayes, 2005)

1.2.10. Diagnosis:

1.2.10.1. Clinical Examination:

Clinical examination, particularly palpation is important for detection and evaluation of breast disease. It remains an extremely useful and practical technique, whether carried out by the physician or by the patient herself. However, both its sensitivity and discriminatory power are

limited. Only 60% of the tumors detected by mammography are palpable the clinical evaluation of axillary lymph node is also fraught with error. Hard lump frequently in the upper, outer quadrant associated with in drawing of the nipple, skin involvement with peaud'orange or frank ulceration and fixation to the chest wall. In elderly the breast became less dense, making the clinical examination easier, so any new lump is likely to represent a malignancy (Heching *et al*., 2002).

1.2.10.2. Mammography:

The wide spread use of mammography has radically changed the diagnostic approach to the breast cancer. Extremely small tumors (1 to 2mm) can be detected with this technique, which relies primarily on the presence of calcification. Used after age 40 years (da Fonte, 2002).

1.2.10.3. Breast Ultrasonography:

Breast ultrasonagraphy has emerged as a valuable examination particularly for determining whether a mass lesion is cystic or solid and state of axilla. It's used before age of 40 years (Mahabir *et al*., 2006).

1.2.10.4. Cytology:

The two methods that have been used to obtain cytological material from breast lesion are aspiration of nipple secretion (which is of very little value) and fine-needle aspiration which is very important with average sensitivity about 87%, the specificity close to 100% (Casciato and Lowitz, 2000).

1.2.10.5. Core Needle Biopsy:

Core needle biopsy can be very useful for documenting the malignant nature of a true neoplasm; its overall sensitivity rate is between 75-90%. The use of core needle biopsy has increase in recent years to the decrease

in the use of open biopsy with frozen section (Casciato and Lowitz, 2000).

1.2.10.6. Open Biopsy and Frozen Section:

Open biopsy from breast lesion are usually of excisional type when the tumor measures 2.5 cm or less and incisional type for larger neoplasm. The procedure is highly accurate, the false-positive rate is essentially zero, the false negative rate is less than 1%, and the number of deferred diagnosis is less than 5% (da Fonte, 2002).

1.2.11. Prognosis of breast cancer:-

There are many prognostic factors associated with breast cancer: staging, tumor size and location, grade, whether disease is systemic (has metastasized, or traveled to other parts of the body), recurrence of the disease, and age of patient. Stage is the most important, as it takes into consideration size, local involvement, lymph node status and whether metastatic disease is present (Hulka and Stark, 2005). The higher the stage at diagnosis, the worse the prognosis. Larger tumor, invasiveness of disease to lymph nodes, chest wall, skin or beyond, and aggressiveness of the cancer cells raise the stage, while smaller tumors, cancer free zones, and close to normal cell behavior (grading) lower it. Grading is based on how cultured biopsied cells behave. The closer to normal cancer cells are the slower their growth and a better prognosis. If cells are not well differentiated, they appear immature, divide more rapidly, and tend to spread. Well differentiated is given a grade of 1, moderate is grade 2, while poor or undifferentiated is given a higher grade of 3 or 4(depending upon the scale used) (Osteen, 2001).Younger women tend to have a poorer prognosis than post-menopausal women due to several factors. Their breasts are active with their cycles; they may be nursing

infants, and may be unaware of changes in their breast. Therefore, younger women are usually at a more advanced stage when diagnosed. The presence of estrogen and progesterone receptors in the cancer cell, while not prognostic, is important in guiding treatment, those who do not test positive for these specific receptors will not respond to hormone therapy (Berkey and Hutter, 1999).

1.2.12. Treatment of breast cancer:-

1. Surgery

Mastectomy is usually advised in patients at increased risk of loco-regional recurrence (in the breast or draining lymph nodes) with BCT and these risk factors include larger tumor size, multicentricity, an extensive intra-ductal component or extensive lymph vascular invasion (White *et al*., 2008). Young age is also a risk factor for loco-regional recurrence, and the place of BCT in young women, particularly those, 35 years old is controversial as they appear to be at higher risk of local recurrence than older women (Schwartz *et al*., 2006). BCT is the standard of care for early breast cancer, and is unlikely to compromise survival in young women (Kroman *et al.,* 2004). However, the relative risks and benefits of BCT versus mastectomy in very young women remain uncertain since this population has not been well represented in large RCT. In addition, as up to 10% of very young women (Malone *et al.,* 2000) may have an inherited predisposition to breast cancer (even in the absence of a family history), unilateral or bilateral mastectomy may be a good option.

2. Chemotherapy

Even when tumors are removed by surgery, microscopic cancer cells can spread to distant sites in the body. In order to decrease a patient's risk

of recurrence, many breast cancer patients are offered chemotherapy. Chemotherapy is the use of anti-cancer drugs that go throughout the entire body to eliminate cancer cells that have broken off from the breast tumor and spread (Spillman and Bowcock, 2006). Many factors go into determining whether an individual patient should have chemotherapy. Generally, patients with late stage disease need chemotherapy; however, chemotherapy can be beneficial even for patients with early-stage disease. Individual factors such as age, overall health, and biologic properties of a woman's breast tumor may go into decisions regarding whether or not she should have chemotherapy. There are many different chemotherapy drugs, and they are usually given in combinations for 3 to 6 months after you receiving surgery. Depending on the type of chemotherapy regimen receive, may be got every 2 to 4 weeks. Most chemotherapy used for breast cancer is given through a vein, so need to be given in an oncology clinic. Drugs that are commonly used in breast cancer treatment include adriamycin (doxorubicin), cyclophosphamide, and taxanes. Generally, chemotherapy is given after surgery for early-stage breast cancer. Sometimes, chemotherapy may be given before surgery to shrink large tumors and allow surgery to be more effective. For patients with stage IV disease, chemotherapy may be given without surgery, and a variety of different agents may be tried until a response is achieved (White *et al*., 2008).

3. Radiotherapy (RT):

Radiotherapy significantly reduces loco-regional recurrence which is associated with increased morbidity and potentially increased mortality. RT is recommended to all women after BCT and significantly reduces local recurrence from about 26% without radiation to 7% with radiation. There is evidence that a radiation boost to the tumor bed is of benefit in young women and further reduces the risk of local recurrence after BCT.

Radiation is also advised following mastectomy to all patients who have a high risk of local relapse, including those with tumors .5 cm and/or those with at least four positive nodes or extensive lymphovascular permeation. The role of RT in women with 1–3 involved axillary nodes is currently being addressed in clinical trials, but there is evidence to support its role particularly in higher risk patients. Age ,45 years, .25% of nodes positive, medial tumor location and estrogen receptor (ER) negative (ER–ve) status have been found to be statistically significant independent factors associated with greater local recurrence, meriting consideration and discussion of post-mastectomy RT (Kroman *et al.,* 2004).

Hormonal Therapy

When the pathologist examines a tumor specimen, may determine that the tumor is expressing estrogen and/ or progesterone receptors. Patients whose tumors express estrogen receptors are candidates for therapy with estrogen blocking drugs. Estrogen-blocking drugs include Tamoxifen and a family of drugs called aromatase inhibitors. These drugs are delivered in pill form for 5 - 10 years after breast cancer surgery. These drugs have been shown to drastically reduce risk of recurrence if tumor expresses estrogen receptors (Spillman and Bowcock, 2006).

4. Biologic Therapy

The pathologist also examines tumor for the presence of HER-2 over expression. HER-2 is a receptor that some breast cancers express. A compound called Herceptin or (Trastuzumab) is a substance that blocks this receptor and helps stop the breast cancer from growing. Patients with tumors that express HER-2 may benefit from Herceptin, and this should be discussed with a medical oncologist when the treatment plan is decided upon (Kroman *et al.,* 2004).

Chapter Two

Materials

And

Methods

Chapter Two
Subjects and Methods

2.1. Subjects:-

2.1. Patients:-

This study carried out 25 fresh biopsy tissue from breast cancer (below 40 years) the mean age was 32.5 years. Samples were collected prospectively from Al-Kadhymia Teaching Hospital through the period between 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 first full term pregnancy and pattern of menstrual cycle. The breast cancer women were interviewed and questioned according to special form (Appendix -I). Confirmatory diagnosis of breast cancer and it differentiation was identified by histopathological examinations.

2.1.2. Control:

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.

2.1.3. 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

2.1.4. 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)	Promega	_____
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	linear chemical	_____

2.1.5. Reagents:-

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

2.1.5.1. Isopropanol:-

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

2.1.5.2. 70% Ethanol:-

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

2.1.6. RIA (radio immuno assay) estradiol kit:-

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

2. 1.7. 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

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

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

2.1.8.1 1X TBE buffer (Tris/Borate/EDTA) electrophoresis buffer

2.1.8.2. 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**.**

2.1.8.3. 6X loading buffer.

2.2. Methods:-

2.2.1. Samples Collection:

2.2.1.1. Blood sampling

Three to five ml of blood was collected in plain tubes from 15 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.

2.2.1.2. 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 first full term pregnancy, menstrual cycle and family history of breast cancer and another type of cancer (appendix-1).

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

2.2.2. Laboratory work:-

2.2.2.1. 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.

2.2.2.2. Lipid profile study of blood samples by linear chemical/Spain:-

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 ⟶ **Cholesterol + Fatty acids**

Cholesterol + O_2 ⟶ **Cholestenone + H_2O_2**

4-AA + Phenol ⟶ **Quinoneimine + $4H_2O$**

A. Total Cholesterol:-

- 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.

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

B. 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.

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

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

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

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

- 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

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

C. 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**.**

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

2.2.4. Molecular Study of Tissue Samples: -

2.2.4.1. 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 65C 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.

2.2.5. 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).

2.2.5.1. Estimation of DNA concentration (Sambrooke and Russell (2001).

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.

2.2.5.2. 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:-

$$\textbf{DNA purity} = \textbf{O.D}_{260} / \textbf{O.D}_{280}$$

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

2.2.6. 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 and Russell (2001).

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.

2.2.7. 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 (0.05 units/ml).
- $MgCl_2$ (4Mm).
- dNTPs (dAtp, dCTP, dGTP , dTTP), (0.4 mM of each).

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 P2 P3	5'GGTTGGCAGCAATATGTGAA'3 5'GCTGACTTACCAGATGGGACTCTC '3 5'CCCAAATTAATCACTCTTGTCGTGACTTACCAGATGGGACAGTA'3	 335bp 354bp
2*BRCA1 538 insC	P4 P5 P6	5'GACGGGAATCCAAATTACACAG'3 5'AAAGCGAGCAAGAGAATCGCA'3 5'AATCGAAGAAACCACCAAAGTCCTTAGCGAGCAAGAGAATCAC C3	 271bp 295bp
3*BRCA2 6174del T	P7 P8 P9	5'AGCTGGTCTGAATGTTCGTTACT '3 5'GTGGGATTTTTAGCACAGCTAGT '3 5'CAGTCTCATCTGCAAATACTTCAGGGATTTTTAGCACAGCATGG'3	 151bp 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

3) DNA ladder:-

DNA ladder was used for DNA molecular size estimation. It was provided by Fermentas Company / Lithuania, with concentration of 0.5µg/µl. This was supplied in buffer composed of 10 mMTris HCL (pH7.6), 1mM EDTA. The DNA ladder contained 11 fragments (in base pair) as follow: 1500,1000,900,800,700,600,500,400,300,200 and 100.

2.2.8. PCR Reaction: - (pak *et al*., 2008).

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.

2.2.8.1 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

2.2.8.2. 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.

2.9. 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

3.1. Risk Factors

3.1.1. Age:

In Eastern societies, Breast cancer is at the top among all the malignancies seen in Iraqi females, comprising of 21.8%. In addition, breast cancer in young Iraqi's women is a crucial problem, with the proportion of young age-onset breast cancer much higher than in western countries. According to the 2008 annual report of Iraqi National Cancer Registry, breast cancers that developed before the age 40 accounted for 36.4% of all female breast cancers compared with only 6.5% in USA (Registry NC:, Cancer Incidence Report Iraq 2008).

Results presented in this work had shown that women (25 cases of breast cancer) were less than 40 years of age, as presented in table (3-1) which showed the distribution of cases according to their age, where the ages of breast cancer women were ranging from 22-39 years old and the mean age was 32.5 years old. So the distribution of patients was significantly occurred between 30-39 years old. While the control group was included 10 normal healthy women all of them were under 40 years old as shown in the table (3-1). Also the percentage of genetic mutations (deletion or insertion) in breast cancer women was 62% distributed as follows: 20 % BRCA1185delAG, 24 % BRCA15382insC, 8 % BRCA21674 and 48 % were normal. For control samples the percentage was 100% normal.

Table (3-1) Distribution of cases and controls according to their ages.

Patients age	Cases		Mutant genotype	Normal genotype	Control	
	No.	%	No.	No.	No.	%
>30	8	32	5	3	5	50
≤30	17	68	9	8	5	50
Total	25	100	14	11	10	100

The results of present study came in agreement with Peto *et al*., (2008) who demonstrated that about 35–45% of Eastern breast cancer women aged less than 40 years are likely to have germ-line BRCA1 or BRCA2 mutations. So that several observations which line with present study and were suggested that breast cancer in young women behave differently compared with breast cancer in middle-aged and elderly women. Hereditary breast cancer is associated with young age strongly which caused disease appearing, where mutations in the BRCA1 and BRCA2 genes are believed to be responsible for around 90 % of hereditary cases of breast cancer in young women. As well as these young women who carriers of BRCA1 or BRCA2 mutations are more likely to present with tumors with high grades of malignancy compared to age-matched controls (Winchester, 2009). Also there are similar results reported by Choi *et al.,* (2004) who showed that percentage of BRCA mutations in

Korean women with breast cancer at a young age (< 40 years) is about 40-50.

3.1.2. Family History

Results indicated in table (3-2) shown that 16 (64%) patients had have family history, 8 0f them had have strong family history while the other patients 9 (36%) have no family history. As well as the control individuals all were with no family history. The results showed that patients with family history had mutations in BRCA1 or BRCA2 genes, except two patients had family history but with no mutated BRCA1 (185del AG, 5382 ins C) or BRCA2 (6174del T) genotype because the family history of these 2 patients was not strong enough to be cause inherited cancer or may be owners other mutations in BRCA genes or other mutation in other genes.

Table (3-2) Association between family history and genotype of BRCA mutation in breast cancer patients and control.

samples	family history	Total No	Normal genotype	Genetic mutation
patients	Yes	16	2	14
	None	9	9	0
Total		25	11	14
control	Yes	0	0	0
	None	10	10	0
Total		10	10	0

These results agreed with Berman *et al.,* (2007) who reported that BRCA1 and BRCA2 are major genes related to hereditary breast cancer. Women who had inherited certain mutations in these genes exhibited a high risk of developing breast cancer during their lifetimes. Also he reported that presence of BRCA1 or BRCA2 in as many as 87% of the cases. Also other observations were reported by Colditz *et al* (2009) was agreeing with results of this study who reported that a family history of breast cancer increases with presence the family history. However, women with at least one first-degree relative with breast cancer have 2 to 4 times the risk of developing breast cancer. Approximately 45% to 60% of breast cancer cases are caused by known mutations in cancer susceptibility genes BRCA1 and BRCA2. These mutations are inherited in an autosomal-dominant manner (Claus *et al.,* 2000). Although the prevalence of BRCA1 mutations in the general population was estimated to be 1 in 100 persons and the prevalence of BRCA2 is unknown but

when two genes are inherited together they can lead to a significant increase in breast cancer risk and the incidence percentage reaches to 70% (Scully *et al.,* 2008). More recent studies had evaluated the rate of occurrence of BRCA1 and BRCA2 mutations and breast cancer in women with such a strong family history of breast cancer, which were more like women in the general population. These studies had estimated that from 36% to 68% of the women with BRCA mutations in the general population would be expected to have breast cancer before age 40 (Stuppia *et al* 2003). Also there was a considerable country-to-country difference in the proportion of cases of breast cancer in breast cancer families that were due to BRCA1 or BRCA2. Almost 80% of cases with a family history of breast cancer (familial cases) in Russia had BRCA1 mutations whereas in other European countries, less than 30% of these familial cases were due to mutations in this gene. In the United States and Canada about 40% of the familial cases were due to BRCA1. On the other hand in the Iceland had a high percentage (64%) of breast cancer families affected by BRCA2 mutations. In the United States and Israel approximately 25% of breast cancer families were due to BRCA2 mutations; in most other countries the proportion is below 20 (Claes *et al* ., 2007).

3.1.3. Menstrual cycles:

Female menstrual cycle characterized by cyclic changeable levels of many sex hormones and this cyclical change play a crucial role in different functions regarding the production and its related conditions. Up to this fact, the serum levels of these hormones seemingly share the carcinogenesis of different human cancers including breast cancer (Gregory *et al*., 2007). Epidemiologic studies have consistently found that the number of lifetime menstrual cycles is associated with breast cancer risk (Bernstein *et al*., 2006). The numbers of menstrual cycles

contribute to exposure to the ovarian hormones that accompany each cycle. It has been suggested that a reduced number of lifetime ovulatory cycles may reduce the risk of breast cancer. Each menstrual cycle is divided into a follicular phase and a luteal phase. In the follicular phase, progesterone levels are low and estrogen levels increase in anticipation of ovulation. In the luteal phase, both progesterone and estrogen are elevated. The length of the luteal phase remains relatively constant, whereas the length of the follicular phase can vary dramatically .Therefore; women with longer menstrual cycles spend more time in the follicular phase than in women with shorter menstrual cycles(Michael *et al*., 2002).

The current study had indicated that 16 cases (64%) of samples have got different types of menstrual disturbance; others 9 cases (36%) of samples have normal, regular menses in addition to 10 control individuals as shown in table (3-3)

Table (3-3) Distribution of breast cancer women according to menstrual cycles.

Patients				Control		
Age	**No.**	**disturbance**	**normal**	**No.**	**disturbance**	**normal**
< 30	**8**	**5**	**3**	**5**	**1**	**4**
≥ 30	**17**	**11**	**6**	**5**	**3**	**2**
Total	**25**	**16**	**9**	**10**	**4**	**6**

A study concluded by Titus-Ernstoff and colleagues at (2001) concluded that there was evidence that menstrual cycles were associated with greater breast cancer risk. Specifically, longer cycles (greater than 28 days) were appeared to increase risk.

3.1.4. First full term pregnancy:

Chie, (2000) who acknowledged that a first full term pregnancy (FFTP), especially an early FFTP before age 24, substantially decreases the long-term risk of breast cancer. Conversely, experts agree that the older a woman is when she has her FFTP, the greater her breast cancer risk (Bernstein *et al*., 2006). The biological reason is that her cancer-susceptible breast is exposed to increased levels of estrogen during every menstrual cycle (Lambe and Ekbom, 2005).

The present study was included 25 patients, 3 patients was single, other patients (22) patients all of them were married. From married women 16 cases (80%) have the first full pregnancy >25, 4 cases (20%) have first full pregnancy <25 years. Two patients were nulliparous.

Present study came in agreement with Dorothy and Pathak, (2007) who reported that increase risk with increasing age at first full term pregnancy which has been can be attributed to the length of the period when the breast susceptible to carcinogen. However, many reports have supported the importance of age at first full term pregnancy (FFTP) as determinant of breast cancer risk, applied multiple logistic regression analysis to a case-controls study conducted in seven area of the world and has found each 5-year increase in age at first full-term pregnancy was associated with increasing of breast carcinoma (Antoine *et al*., 2004). The inverse relationship between age at first full-term pregnancy and breast cancer risk may relate to full differentiation of mammary gland epithelium at the

first full-term pregnancy, which makes the cells less susceptible to carcinogenic changes (Neuhausen *et al*., 2009).

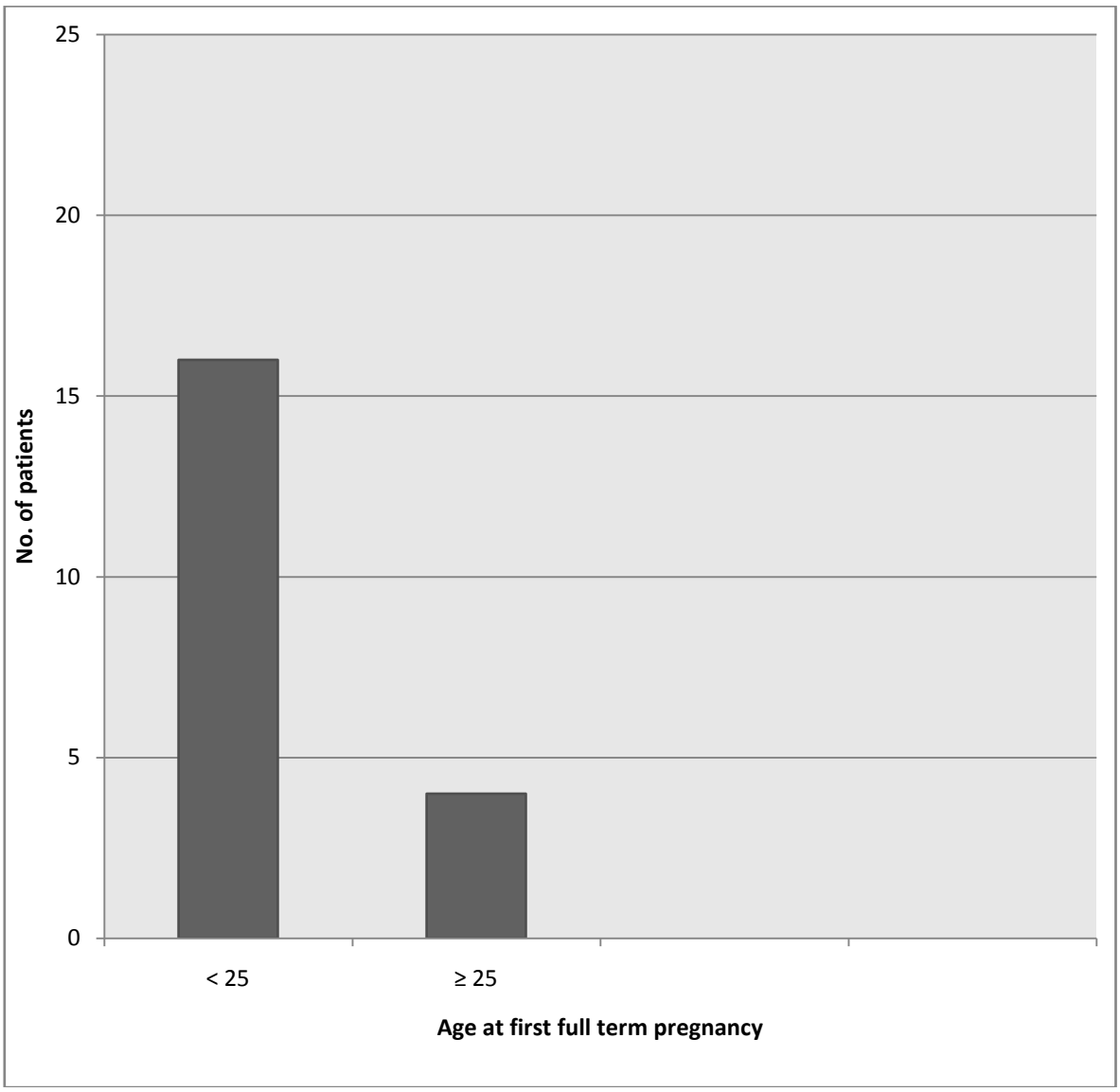


Figure (3-1) distribution the patients according the age at first full term pregnancy.

For women who delay a FFTP until after age 25, there is a slight, short-term risk of breast cancer that disappears within 15 years after childbirth.

3.2.1. Estrogen

The etiology of breast cancer is thought to involve a complex interplay of genetic, hormonal and environmental factors that influence the physiological status of the host. However, there are substantial experimental, epidemiological and clinical evidences, which show that breast cancer risk is influenced by endogenous sex-steroid hormones (Hulka and Stark, 2005).The present study includes 15 patients blood samples were taken from them, as well as to 10 individuals as control with patients. This result clarify that 11 (73.3%) cases was prevalence increased estrogen level, others 4 (26.7%) patients showed normal estrogen level. While control individuals all of them have had a normal estrogen levels .The result of this study ensure that estrogen level has important effect on incidence of breast cancer in premenopausal (< 40 years) women ($P \leq 0.05$) as shown in table (3-4).

Table (3-4) distribution of cases according the estrogen levels.

	Elevated		**decreased**		**Normal**		**Total**
cases	**No.**	**%**	**No.**	**%**	**No.**	**%**	
	11	73.3%	0	0	4	26.7	15
control	0	0	0	0	10	100%	10

Current results agree with Schairer *et al*., (2007) who found it is clear that there is a correlation between high estrogen exposure and high breast cancer risk during the years when women have functional ovaries. Also agreement with Titus-Ernst off *et al*., (2001) who reported that an increase in exposure to circulating estrogens during premenopausal years really caused breast cancer. A hypothesis is proposed that estrogens might play an important role in affecting breast cancer risk. On one hand, there is evidence to indicate that estrogens might serve as pre initiators, initiators, and promoters of breast cancer. Generally, association estrogens with promotion of the growth of existing malignancies in the breast as showed in figure (1-2) (Liehr, 2000). Estrogen exposure, particularly during puberty and young adulthood, increases the penetrance of breast cancer in germ-line BRCA1 mutation carriers. Although approximately 70% of women who carry a germ-line BRCA1 mutation will develop breast cancer before age 40 years (Loman *et al*., 2001).

Women possessing germ-line mutations in BRCA1 are particularly susceptible to breast cancer as a result of pregnancy (Jernstrom *et al.,* 1999). the BRCA1 alleles is lost due to a mutation, as is the case in familial breast cancer, estrogens might be more likely to cause genomic instability than if both alleles were functioning normally. This would mean that estrogen exposure, particularly during puberty and young adulthood, increases the penetrance of breast cancer in germ-line BRCA1 mutation carriers (Ford *et al*., 2008). In addition to the fact that BRCA1 seem to be induced by estrogens and play a role in DNA repair, their relationship is shown to be more than merely general. Several lines of evidence indicate that estrogen associated with breast cancer in BRCA1 mutation carriers. Premenopausal women exposed to elevated estrogen

levels may also exhibit an increase in BRCA2 expression (Spillman and Bowcock, 2006).

Table (3-5) Total number of cases with their hormonal levels.

Case No.	Age	Menstrual status	Estrogen Pg/ml
1	30	Disturbed	223
2	35	Regular	222
3	28	Disturbed	215
4	32	Disturbed	217
5	39	Regular	120
6	33	Regular	200
7	27	Disturbed	275
8	36	Disturbed	228
9	36	Regular	121
10	33	Disturbed	223
11	34	Disturbed	240
12	29	Regular	284
13	35	Regular	68
14	38	Regular	300
15	32	Disturbed	240

Mean $\pm$ S.D =217.87 $\pm$ 77.38

Table (3-6) Total number of control individuals with their hormonal levels.

Case No.	Age	Menstrual status	Estrogen Pg/ml
1	21	Regular	72.50
2	27	Regular	90.30
3	35	Disturbed	113.40
4	34	Regular	78.70
5	28	Disturbed	66.80
6	39	Disturbed	120.1
7	29	Regular	99.80
8	36	Disturbed	68.00
9	29	Regular	59.74
10	37	Regular	89.32

Mean $\pm$ S.D =128.5 $\pm$ 30.23

3.3. Lipid profiles

Serum levels of various lipid parameters i.e. triglycerides (TG); total cholesterol (TC) and high density lipoprotein (HDL) were measures in a total of a 15 histological proven cases of breast cancer along with 10 control women. The mean levels of serum triglycerides, total cholesterol and high density lipoprotein were found to be not significantly different in breast cancer cases as compared to controls. The findings of present study on premenopausal women were similar to Caleffi *et al.,* (2009) and

Sharma and McGuire, (2001) who failed to show any change in TG, TC and HDL. Levels of TG, TC and HDL were normal in premenopausal patients while of TG, TC and HDL were increase in postmenopausal patients (Atalay *et al*., 2004). On other hand, Brown *et al*., (1997) reported a significant increase in TC, TG and HDL level in premenopausal patients. Also Furberg *et al*., (2004) demonstrated an increased risk of premenopausal breast cancer by increasing or decreasing serum HDL, TG and TC among overweight and obese women.

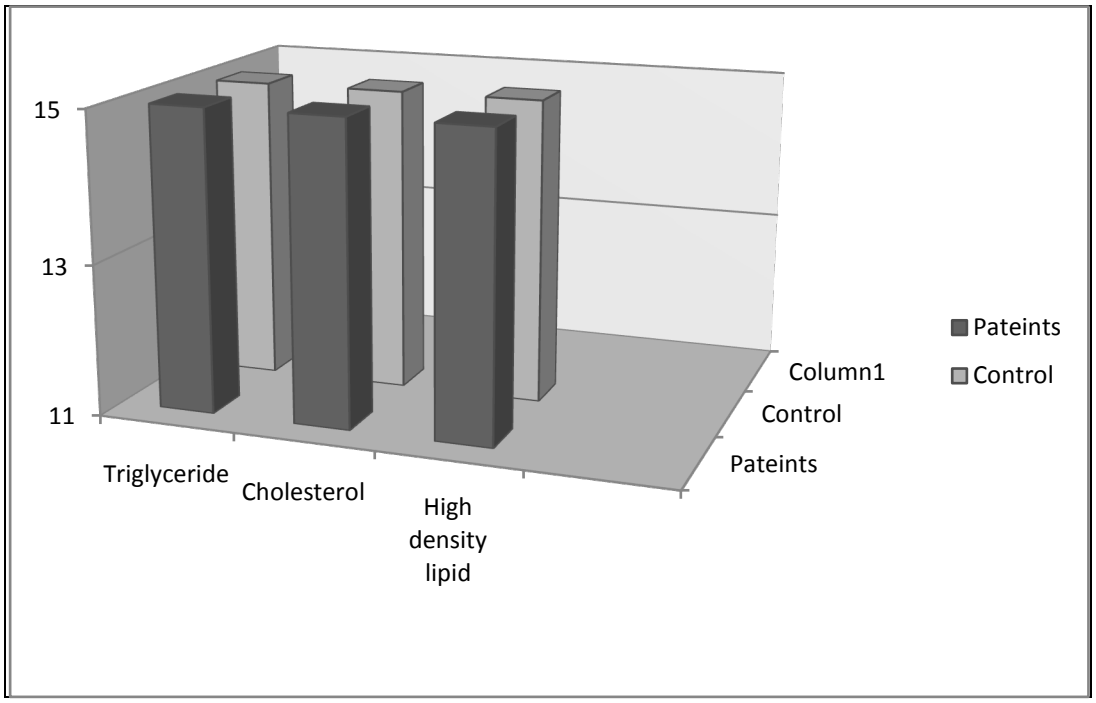


Figure (3-2) Distribution patients and control women according lipids values.

3.4. 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.

3.4.1. 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 this method was found not to be suitable for the purpose of experimental work designed in this research. Therefore another method of DNA extraction was followed, using extraction kit.

The PCR-amplified exons 2 and 20 of BRCA1 gene and exons 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. 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 (Mansukhani *et al*., 1997).

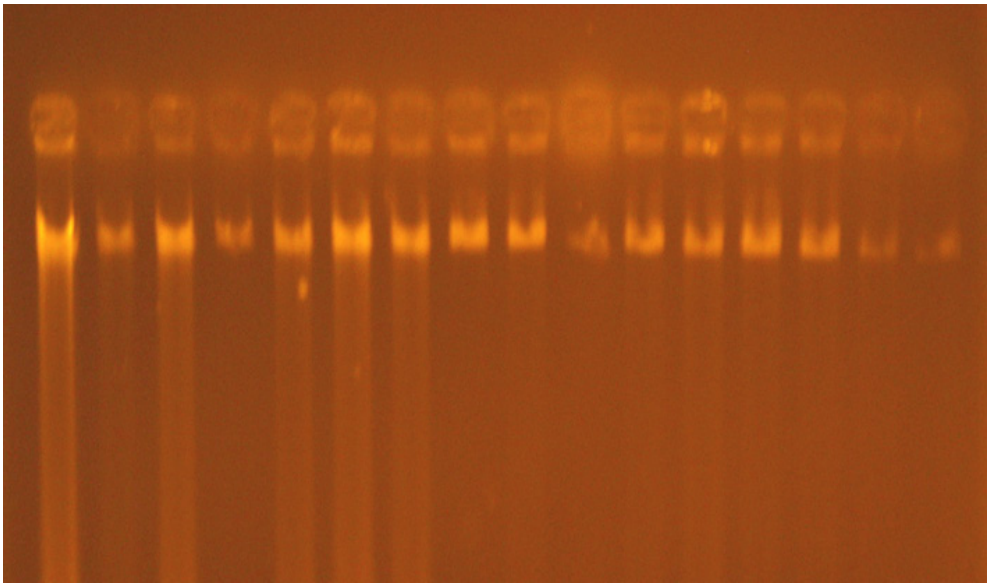


Figure (3-2): 0.8% agarose gel electrophoresis of DNA samples. (100V, 30 min.)

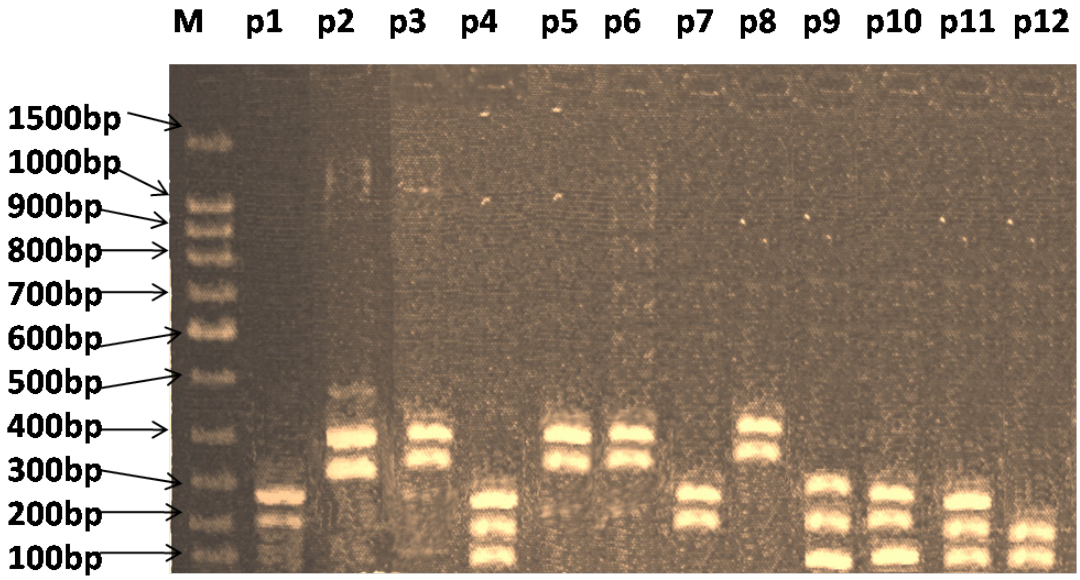
3.4.2 Genetic Factor:

Identification and characterization of genetic alterations such as deletion, insertion and amplifications in the genome that predispose to the development of breast cancer are of at most importance in gaining a complete understanding of the exact molecular events involved in the development of tumorgenesis, it will also provide new targets for early diagnosis and facilitate treatment design (Singh and Roy, 2001).

The current study included 25 patients with invasive breast cancer and 10 healthy women as controls. All patients were analyzed for constitute BRCA mutation. The genomic DNA was used to detect the mutations by using 9 primers. The results of this study showed that the percentages of BRCA1 (185delAG, 5382insC) and BRCA2 (6174delT) were: 36%, 12% and 8% respectively as shown in table (3-9). This mean that the mutation in BRCA1 185delAG was more than that in others. More of the carriers of BRCA1 185delAG have a strong family history.

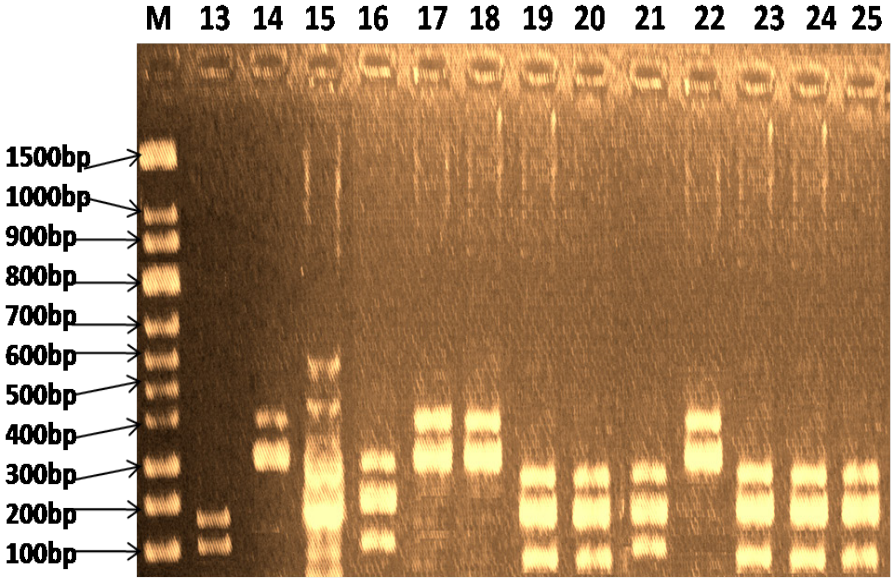
Table (3-9) Association between age and BRCA mutations.

Age of patients	Total	BRCA1185 delAG	BRCA15382 ins C	BRCA26174 del T	No mutation
<30	8	3	1	1	3
≥30	17	6	2	1	8
Total	**25**	9(36%)	3(12%)	2(8%)	11(44%)
Control					
<30	5	0	0	0	5
≥30	5	0	0	0	5
Total	**10**	**0**	**0**	**0**	**100%**



(3-3) Electrophoresis of PCR products on agarose gel 2% (100 volt / 45min).

354bp =185del AG band **wiled type**=335
295bp = 5382insC band **wild type**=271
171bp = 61742delT band **wild type**=151
Lane 1, 7, 15**:** 5382insC BRCA1 **P=** Patient
Lane 2, 3**,** 5, 6, 8**:** 185del AG BRCA1
Lane 12**,** 13**:** 61742delT BRCA2
Lane: 4, 9, 10, 11.
M: DNA marker (100 -1500bp).



(3-4) Electrophoresis of PCR products on agarose gel 2% (100 volt / 45min).

354bp =185del AG band wiled type=335

295bp = 5382insC band wild type=271

171bp = 61742delT band wild type=151

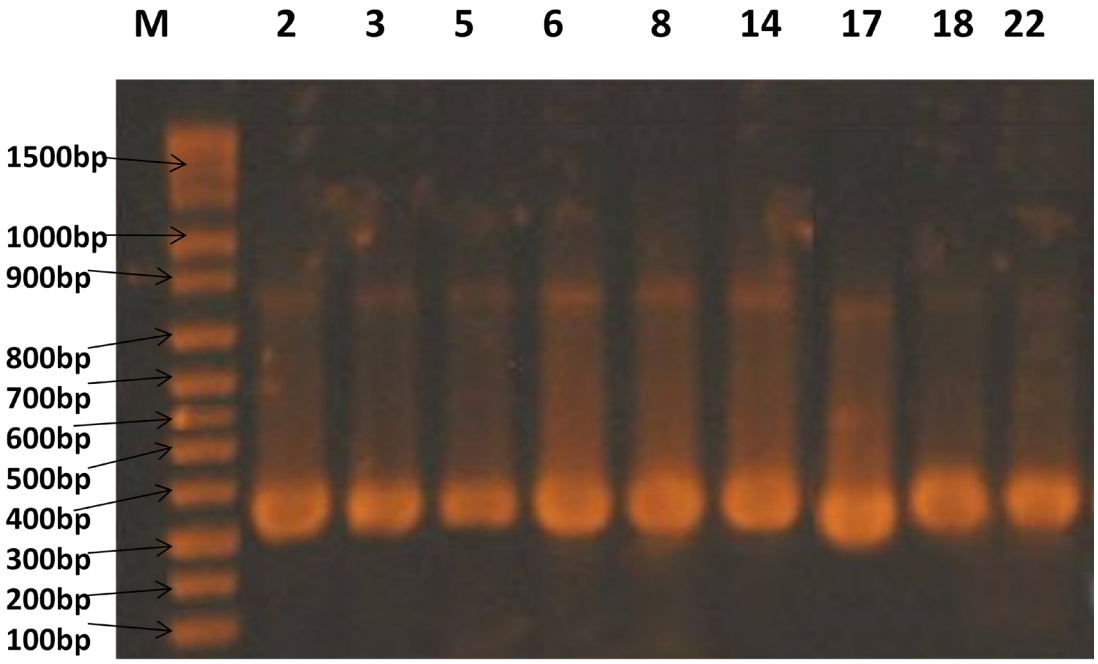
Lane 15**:** 5382insC BRCA1

Lane 14, 17, 18, 22**:** 185del AG BRCA1

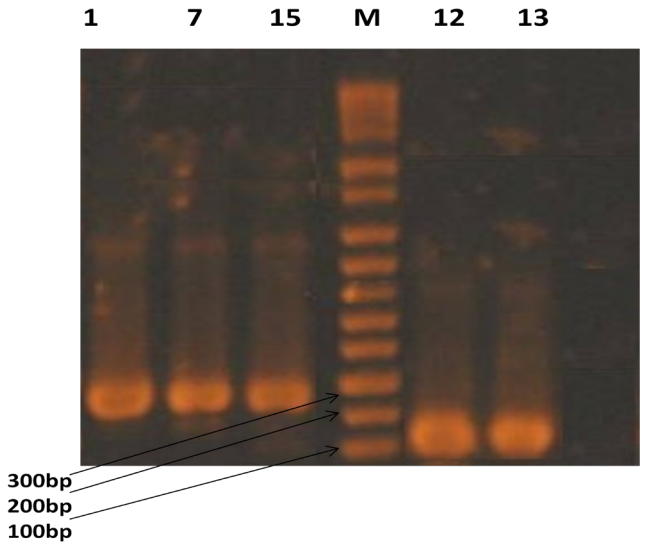
Lane: 16, 19, 20, 21, 23, 24, 25.

M: DNA marker (100 -1500bp).

P= Patient



(3-5) Agarose gel electrophoresis (2%) showing BRCA1 185delAG detected by (100 V., 45 min) PCR Amplification, lane 2, 3, 5,6,8,14,17, 18, 22 show mutant type. M: DNA marker (100 -1500bp).



(3-6) Agarose gel electrophoresis (2%) showing BRCA1 5382insC and BRCA2 6174del T detected by (100 V., 45 min) PCR Amplification, **lane** 1, 7,15 show mutant type for 5382ins C and **lane** 12, 13 show mutant type for 6174del T . **M**: DNA marker (100 -1500bp).

Seventeen to 30% of all breast cancers were related to inherited genetic mutations. Mutations in the BRCA 1 gene was the most prevalent inherited mutations. A family history that is suspicious for a BRCA1 mutation includes breast cancer before the age of 40. Other suspicious findings include a family history of breast cancer. Epidemiological studies indicated that BRCA1 mutation carriers had a lifetime risk of breast cancer that is on the order of 60–80% (Fleming *et al*., 2003).

Sobczak *et al*., (2009) suggested that the BRCA1was mutated in 45% of all hereditary breast cancer cases. The spectrum of BRCA1 and BRCA2 mutations has been characterized in different populations worldwide, with significant variation of the relative contribution of these genes to hereditary cancer between populations and examples of population specific founder mutations (BRCA1: 185delAG, 5382insC, BRCA2:6174delT in Jews population) (Szabo *et al*., 2007). In other words, women with an altered BRCA1 or BRCA2 gene are 3 to 7 times more likely to develop breast cancer than women without alterations in those genes (Walsh *et al*., 2006), with very high relative risks for early disease onset (before age 40) of about 30-fold. Researchers discovered the 185delAG mutation in numerous women who do not identify as Jewish or appear to have Jewish ancestry. One large study of Spanish women with breast cancer reported that the 185delAG mutation accounted for 16.7% of all mutations (Goldgar *et al* 2004). Other studies found that the 185delAG mutation constituted 10.1% of all the BRCA1 mutations in Dutch women, 6.5% of mutations in German women, and 3.4% of mutations C in each woman. (Hedau *et al*., 2004) In the United States, 185delAG has been identified as the most common BRCA1

mutation in a sample of Hispanic women in Los Angeles. (Weitzel *et al*., 2005). It has also been found in Hispanic women in Colorado, in Spanish Gypsies, and among South Indian women (Valarmathi *et al* 2004) .Overall prevalence data remain unknown because population-based studies have not been conducted in these groups.

The results came in agreement with (Heching *et al*., 2002) who observed the mutation BRCA1 185delAG dominantly appeared in the substantial proportion of high risk Ashkenazi families. The same mutation was observed in non Jewish population (Warner *et al*., 2001). The BRCA1 185delAG mutation is more frequently than other mutation of BRCA1 gene (Peto *et al*., 1999). In the present study the incidence of BRCA1 mutation was higher than BRCA2 mutation. This seems to phelan *et al*., (2002) who found BRCA2 mutation in 8% of female with family history.

Chapter Four
Conclusions

And

Recommendations

Conclusions:-

1- The study demonstrated that the frequency of BRCA1 mutation (48%) was more than BRCA2 (8%) in women below 40 years.

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

3- 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.

4- Proceeding genetic testing for patient's family by taking blood samples and extracted DNA from them to check spreading of the disease.

5- Research the role of other steroid hormones as well as estrogen hormone in breast cancer risk and determining the risky hormonal status and targeting the therapy.

Appendix

APPENDIX I

Department of Biotechnology/Baghdad 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

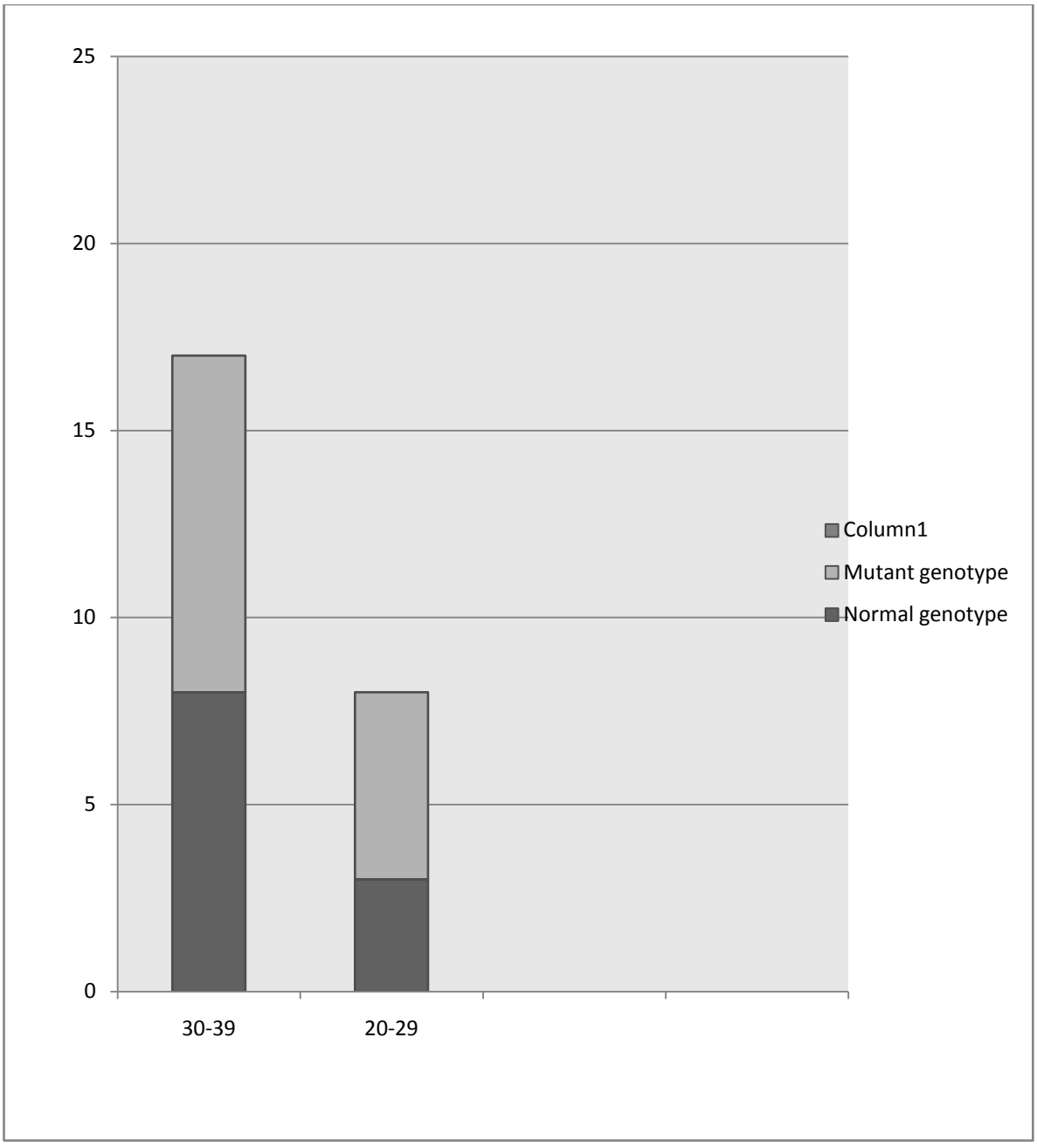
13. Date of Diagnosis _____

14. Affected side: Left Right Both

15. Date of Surgery: _____

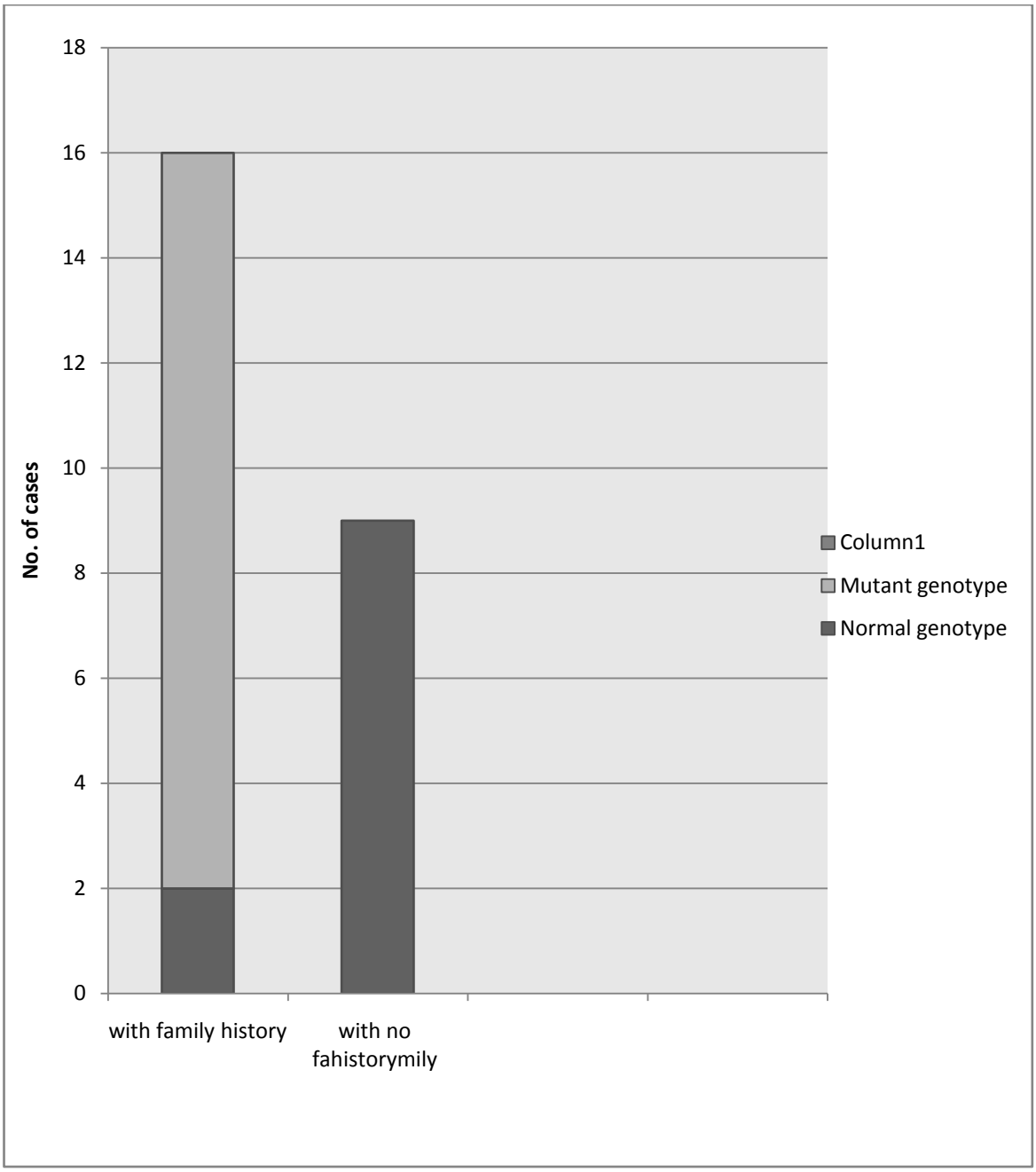
16. Date of Sample _____

APPENDIX II



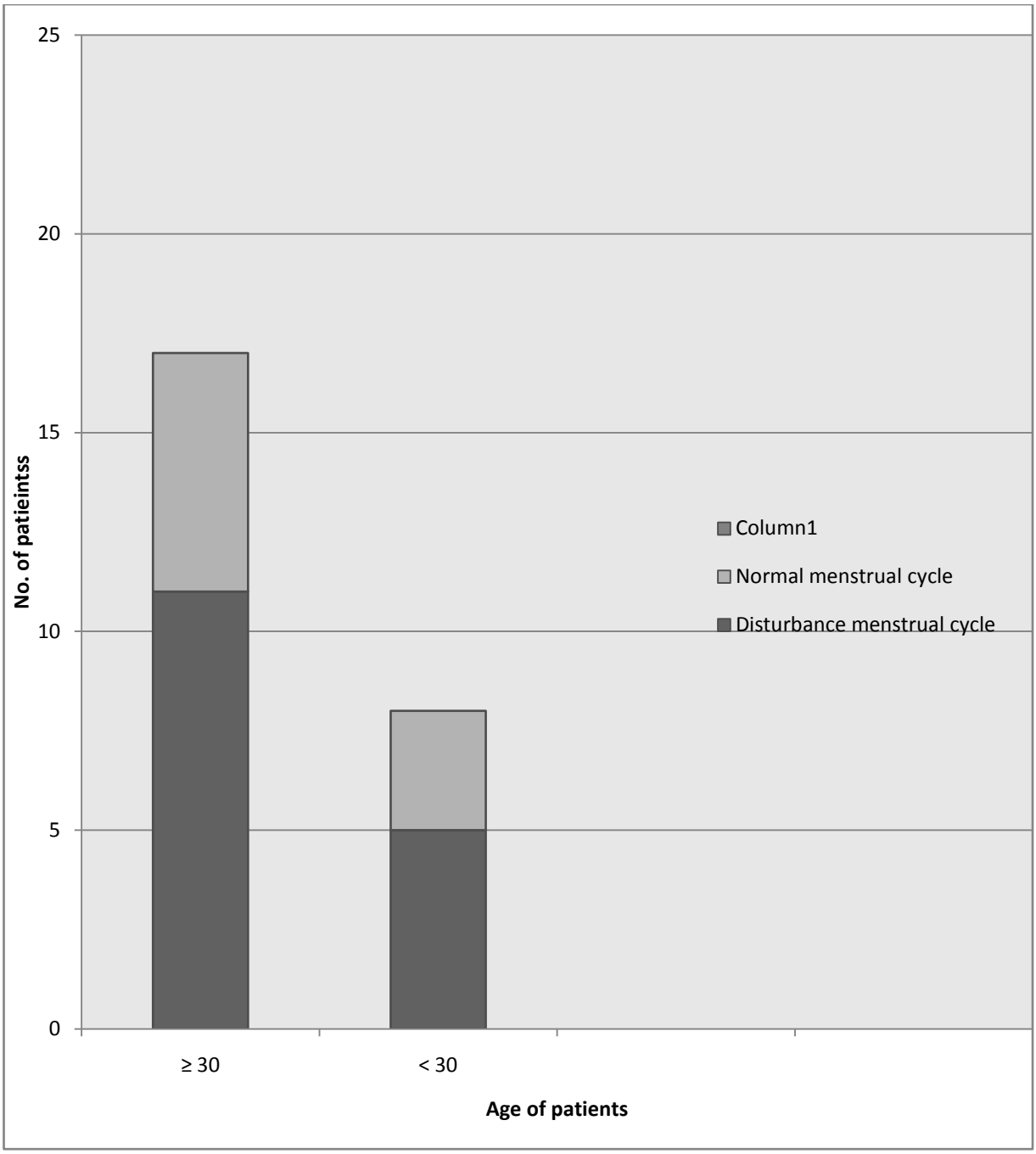
Distribution of cases according to their ages

APPENDIX III



Association between family history and genotype of BRCA mutation in breast cancer women and control.

APPENDIX V



Distribution of breast cancer patients according to menstrual cycles

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الخلاصة

يعد سرطان الثدي السرطان الاكثر شيوعا الذي يصيب النساء في مختلف انحاء العالم .
وقد تناولت الدراسات و البحوث مختلف العوامل المسببة لهذا المرض من اجل التوصل للتشخيص المبكر والعلاج الناجح له. تناولت هذه الدراسة الحالية العلاقة بين التغايرات الوراثة للجينين **BRCA1** و **BRCA2** وعدد من العوامل الخطرة 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 تحت الاربعين سنة. كما تضمنت الدراسة العوامل آلاتية : العمر، العمر عند أول حمل وحالة انتظام الدوره الشهرية وتاريخ العائلة بالنسبة الى سرطان الثدي أو اي نوع اخر من الاورام و هرمون الاستروجين.

اخذت عينات دم من 15 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 هذه 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 73.3% من مريضات المصابات بسرطان الثدي . وتعكس هذه النتائج العلاقة الايجابية 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 الطفرة في جيني **BRCA1** و **BRCA2** ان وجدت.

اظهر التحليل الوراثي ان نسبة الحذف في مريضات سرطان الثدي كانت 36% (9 مريضات) للطفرة **185del AG** الموجودة في جين **BRCA1**، اما نسبة الإضافة للطفرة **5382ins C** الموجودة في جين **BRCA1** للمريضات المصابات بسرطان الثدي 12 % (3 حالات)، بينما وجدت الطفرة الاخيرة **6174 T** في الجين **BRCA2** موجودة في حالاتين بنسبة(8%). واستنتج من هذه الدراسة ان نسبة حدوث الطفرة في جين **BRCA1** 48% هي اعلى من نسبة حدوث الطفرة في جين **BRCA2** 8% في العيّنة من النساء العراقيات المصابات بسرطان الثدي.

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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

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