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Molecular Study of Some Genes Associated With Breast Cancer and Estimation of IL12 in Babylon Province Patients

Submitted to the Council of the College of Science for women University of Babylon In Partial Fulfillment of the Requirements For The Degree Of Master In Biology

By

Rose Adil Hameed AL-Saadi

Supervised by

Assist. Prof.
Dr. Ishraq Abdul Ameer Saleh Al-Maamori

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بِسْمِ اللهِ الرَّحْمنِ الرَّحِيمِ

﴿قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا
إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ ﴿٣٢﴾﴾

صدق اللهُ العلي العظيم

البقرة (٣٢)

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I certify that this thesis entitled " **Molecular Study of Some Gene Associated with Breast Cancer and Estimation of IL12 in Babylon Province Patients**" was prepared by " **Rose Adil Hameed Al-Saadi** "

under our supervision at the Department of Biology College of Science for Women, University of Babylon , as a partial fulfillment of the requirements for Degree of Master in Science of Biology .

Supervisor **Signature**

Assist. Prof. Dr. Ishraq Abdul Ameer Saleh

Department of Biology

College of Science for Women / University of Babylon

In view of the available recommendations , I forward this thesis for debate by the examination committee .

Department Head **Signature**

Prof. Dr. Ekhlas Al-Shareefi

Head of Biology Department

College of Science for Women / University of Babylon

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We, the member of examining committee, certify that we have read the thesis entitled **(Molecular Study Of Some Gene Associated with Breast Cancer and Estimation of IL-12 in Babylon Province patients)** and after examining the master student **(Rose Adil Hameed Al-Saadi)** in its contents in 15/10 /2023 and that in our opinion it is adequate as a thesis for degree of master in Biology with degree (Excellent).

Committee Chairman

Signature:
Name: Dr. Abeer Fauzi Murad Al-Rubaye
Scientific order: Professor
Address: Collage of Science for Women/University of Babylon
Date:

Committee Member

Signature:
Name: Dr. Zainab Mohammed Jasim
Scientific order: Assist. Prof.
Address: College of Biotechnology/Al-Qasim Green University
Date:

Committee Member(Supervisor)

Signature:
Name: Dr. Ishraq Abdul Ameer Saleh
Scientific order: Assist. Prof.
Address: Collage of Science for Women/University of Babylon
Date:

Committee Member

Signature:
Name: Dr. Zainab A. Tolaifeh
Scientific order: Assist. Prof.
Address: Collage of sciences/ University of Babylon
Date:

Date of Examination: 15 / 10 / 20232

Deanship authentication of Science College for Women Approved for the college committee of graduate studies.

Signature:

Name: Dr. Abeer Fauzi Murad Al-Rubaye

Scientific order: Professor

Address: Dean of Science Collage for Women/University of Babylon.

Date:

Dedication

To the sake of Allah, my Creator and my Master..

To my great teacher and messenger, Mohammed (May Allah bless and grant him), who taught us the purpose of life..

To my homeland Iraq..

To my great parents..

To my dear Husband (Ali Shawqi), who supported and trusted me and My beloved kids (Yildiz and Ronza)..

To all my family, my friends and all people whom i loved..

To everyone who aided me in every possible way to make this work see the light..

I dedicate this work..

Rose Adil

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My special and sincere thanks to patients who i take the samples from and may Allah heal them and have mercy on those who died .

ROSE ADIL

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.

This investigation was carried out on 50 patients (all were females) who were confirmatory for breast cancer by histopathological examinations attended to hospital Merjan in Hillah city .

The study was conducted between September 2022 to July 2023 at University of Babylon College of Science for wemen .They were divided according to their clinical end point into:50 patients and 50 of apparently healthy women were used as a control. All women (patients and control) their age between 30-71 years, family history, menopause, marital state were taken into account as risk factors.

The result shows no significant effect for marital state and significant effect for menopause and family history .In addition, find the association between DNA polymorphism of some the selected genes and breast cancer and to investigate the gene mutation that may be related to Breast cancer using , amplification refractory mutation system (ARMS) PCR techniques and restriction fragments length polymorphisms (PCR-RFLP).

It was observed that the means distribution of IL-12 (pg/ml) patient groups and control groups by ELISA. It was clear that IL-12 level has been reduced IL-12 levels in BC patients significantly ($P \leq 0.05$) than control groups .

The present study investigated the relationship between genetic polymorphisms of Breast cancer susceptibility genes 1(*BRCA1*) & Breast cancer susceptibility genes 2 (*BRCA2*) and Fork head box protein

3(*FOXP3*) genes and evaluation of some etiological risk factors among breast cancer patients.

The result shows the distribution of 185delAG mutation of *BRCA1* gene in control and patients Breast cancer. AA 52%, AG 32% and GG 16% in controls and 18%, AG 46% and GG 36% in cases. An increased frequency of homozygote mutant genotypes (GG) were found in patients compared to controls. There was a statistically significant difference in the distribution of allele frequencies in patients and controls (AG: $P<0.005$, OR 0.32 , 95% CI 0.18 to 0.58)

This study found the distribution of *BRCA2* gene in control and breast cancer patients . There are significant difference in AT genotype between patient and control group ($p<0.005$),The A allele is significantly associated with an increased risk of BC

Genotype RFLP technology used in the *Foxp3* gene (rs3761548) which is a polymorphic gene that has a significant impact on breast cancer patients. The *Foxp3* genotype showed critical variety between alleles in patients and control, the AA was showed up in 70% control while it was 22% in patients, CC genotype was more successive in patients (52%) than control (24%). AC genotype was continuous in control (6%) than patients (26%).

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List of Abbreviations

NO.	Abbreviations	Terms
1	ARMS	Amplification refractory mutation system
2	BC	Breast Cancer
3	BP	Base pair
4	BRCA1	Breast cancer 1
5	BRCA2	Breast cancer 2
6	BSA	Bovine serum albumin
7	BSE	Breast Self-examination
8	DNA	Deoxyribonucleic acid
9	EDTA	Ethylene diamine tetra acetic acid
10	ELISA	Enzyme linked immune sorbent assay
11	F	Forward
12	FABG	β-ketoacyl-ACP reductase
13	FNA	Fine Needle Aspiration
14	FOXP3	Forkhead box gene
15	HRP	Horse reddish peroxidase
16	IL-12	Inter Leukien-12
17	MIN	Minutes
18	MRI	Breast Magnetic Resonance Imaging
19	NEB	New England biolab
20	NK Cell	Natural killer cell
21	OD	Optical density
22	OR	Odd ratio
23	P. value	Probability value
24	PCR	Polymerase chain reaction
25	PW Buffer	preheating of domestic water buffer
26	R	Revers
27	RFLP	Restriction fragment llength polymorphism
28	RT	Room temperature
29	TBE	Tris Borate EDTA Buffer
30	WHO	World Health Organization
31	WW	Homozygous wild/wild wild
32	WM	Heterozygous /wild mutant
33	MM	Homozygous mutant /mutant mutant
34	AJCC	American joint committee on cancer

35	EMR	Eastern Mediterranean region
36	SBR	Scarf Bloom Richardson (grading system)
37	HER	Human
38	Ebi3	Epstein Barr Virus induced genes
39	Pst1	Providencia Stuartii restriction enzyme
40	TNM (method to know the stage of breast cancer)	T/ indicate the size of tumer N/indicate the size of tumer in the lumphonode Mstand for metastasis

Chapter one

Introduction

1.Introduction:

Breast Cancer a malignant breast neoplasm, is one of the leading causes of female deaths worldwide accounting for 3.1% annual global increase in developing countries (Jiang *et al*., 2021). Breast Cancer is 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 (Stella *et al*., 2021).

The etiology of breast cancer depends on various epidemiological factors; however, genetic susceptibility plays a major role in the causation of the disease as a small portion of the exposed individuals develop breast cancer (Lee *et al*., 2020) .

Breast cancer is the result of genetic alterations (mutations) in one of genes that may be involved 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 (Cheng *et al*., 2020).

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 (Incorvaia *et al*., 2020).

Forkhead box protein3 *(FoxP3)* gene, which more recently has been implicated in the development of BC (Guo *et al.*, 2020). *FoxP3*, was initially identified as a gene responsible for X-linked autoimmune

diseases in mice and humans (immune dysregulation, polyendopathy, enteropathy, X-linked, IPEX), and a master regulator of the development and function of regulatory T cells (Treg) (Qiu *et al*., 2020).

The role of *FoxP3* in the development and metastatic spread of BC is supported by several lines of evidence. First, the broad expression of *FoxP3* gene in breast epithelial cells, and its down regulation in the mammary cancer tissues. Second, the high rate of *FoxP3* mutations or deletion in the majority of BC samples (Raskin *et al*., 2009).

The complex etiology of the disease comprises an interaction between genetic, hormonal and environmental factors where family history represents a documented risk for its onset. It has been illustrated that the severity of the risk depends on the degree of family involvement, the age and the number of the affected relatives; probably reflecting the interaction between multiple genetic variants and shared environmental exposures among relatives (Dorling *et al*., 2021).

It is widely established that cytokines of the IL-12 family regulate both distinctive and adaptive immune rejoinders. The cytokines of the IL-12 family have been extensively researched in the context of infection and autoimmune disease. There has been a great deal of investigation into how these cytokines impact the immune response to cancer. Likewise, cytokines of the IL-12 family are typically generated by innate immune cells, although adaptive immune cells can also release them depending on the disease or immuneenvironment (Liu *et al*., 2021).

Interleukien-12 may both activate innate (NK cells) and adaptive (cytotoxic T lymphocytes) immunities insusceptibility, it appears to be most suited for tumor immunotherapy. (Tsion & Ephrem , 2023).

Aims of study :-

The aim of this study is detection some of gene related with Breast Cancer such as BRCA1, BRCA2 , FOXP3 and investigated whether immune system impairment manifested by estimation of IL12 in breast cancer patients.

Chapter two

Literature review

2.Litrerature review

2.1 Cancer

Cancer is a category of disorders in which aberrant cells develop and spread uncontrollably. It is possible that if the spread is not controlled, it will result in death (American Cancer Society, 2019a).

Cancer is the main or second leading cause of mortality (defined as death between the ages of 30 and 69 years) in 134 of the 183 nations surveyed, and it ranks third or fourth in another 45 (Cao *et al.*, 2020).

It is likely cancer will become the primary cause of death worldwide as treatments for cardiovascular disease and infectious diseases continue to improve with modern medicine and the continued extension of average life expectancy (Hays, 2022).

Around one in every five men and one in every six women get cancer during their lifetime, with one in every eight men and one in every eleven women dying from it (International Agency for Research on Cancer, 2018).

According to the International Agency for Research on Cancer (International Agency for Research on Cancer, 2020). Iraq had 31,502 new cancer cases in 2018, with an incidence rate of 82.62/100000 people

DNA damage and genomic instability are the primary causes of sporadic (non-familial) malignancies. This genetic instability can present itself on a variety of levels, ranging from basic deoxyribonucleic acid (DNA) sequence mutations to chromosomal anomalies (Ferguson *et al.*, 2015).

By 2040, the anticipated worldwide cancer burden is likely to approach 27-28.4 million new cancer cases per year, up 50% from the estimated cases in 2018 and 47% from the forecast cases in 2020 (Fidler Benaoudia and Bray, 2020).

2.2 Breast cancer

Breast cancer refers to a group of disorders in which cells in the breast tissue divide and alter out of control, resulting in a lump or tumor (Hanna , 2022). Breast cancer can start in a number of places in the breast (Salman , 2021). The lactiferous ducts (ductal carcinoma) that deliver milk to the nipple are where most breast malignancies start. Some begin in the cells of the milk-producing glands' lobules (lobular carcinoma) (Martini *et al*., 2018).

Breast cancer is the most frequent cancer in women of all races and ethnicities, and it is a significant cause of death in women. Early identification is linked to a lower mortality rate (Tkaczuk *et al.*, 2016).

The size of the tumor, evidence of tumor progression to local lymph nodes, and evidence of distant metastases are used to stage breast cancer (primm *et al*., 2022).

2.3 Breast Anatomy

The mammary glands are skin-based sweat glands that have been changed. A pigmented region, the areola, is located just below the center of each breast and surrounds a central projecting nipple (Figure 2-2).

Each mammary gland has 15 to 25 lobes that extend around the nipple on the inside (Marieb and Keller, 2018).

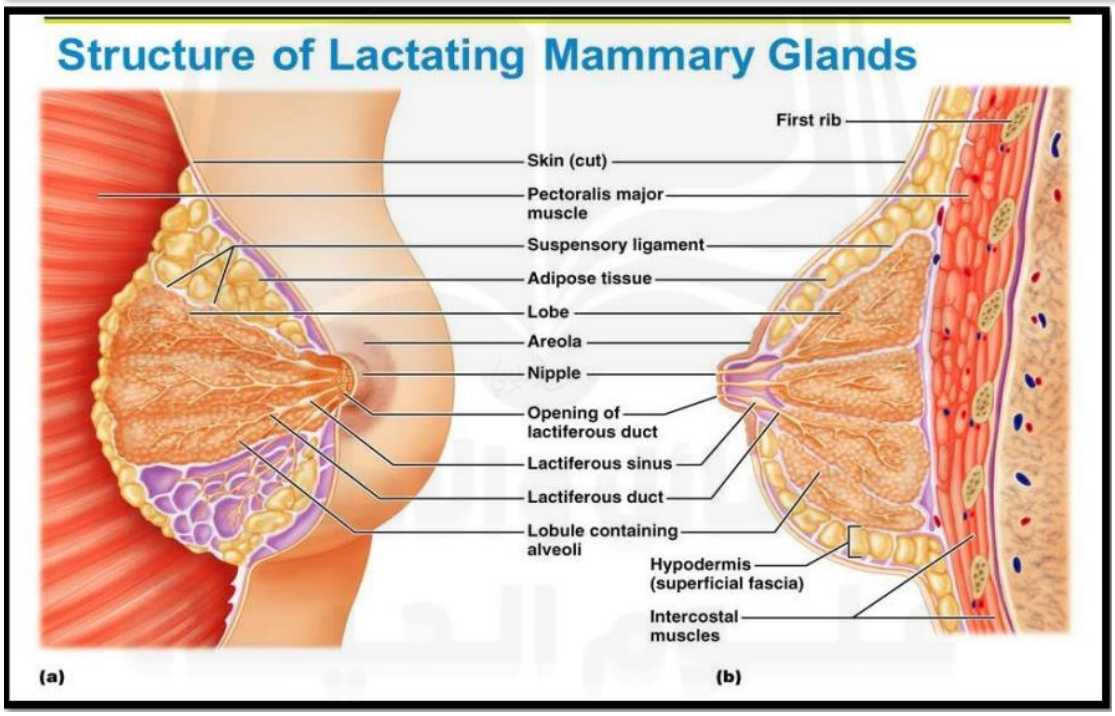


Figure 2-1: Female mammary glands (a) Anterior view (b) Sagittal section (Marieb and Keller, 2018).

Each lobe of the human nipple is made up of 10-100 milk-producing alveoli with a diameter of 0.12 mm. Fat, ligaments, blood vessels, lymphatic vessels and nerves are all found in the stroma and connective tissue that surround the nipple (Bazira *et al*., 2022).

The mammary gland is a complex secretory organ made up of a variety of cell types, including epithelial cells that make up the gland's ductal network, adipocytes that make up the fat pad, vascular endothelial cells that make up the blood vessels, stromal cells, such as fibroblasts, and immune cells. For the most part, the lactiferous duct is lined with cuboidal or columnar epithelium and a conspicuous basement membrane (Figure 2-3) .

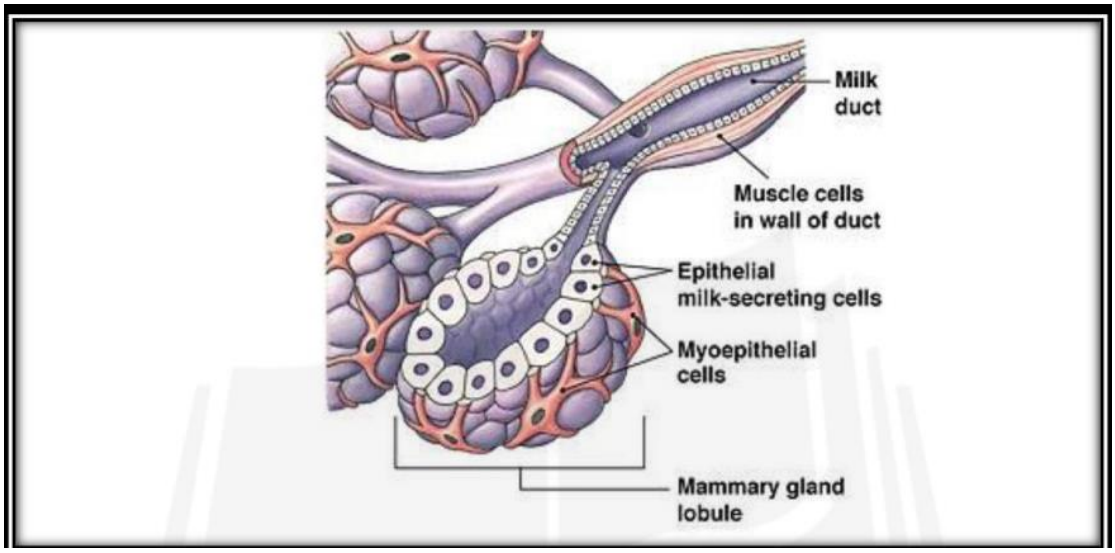


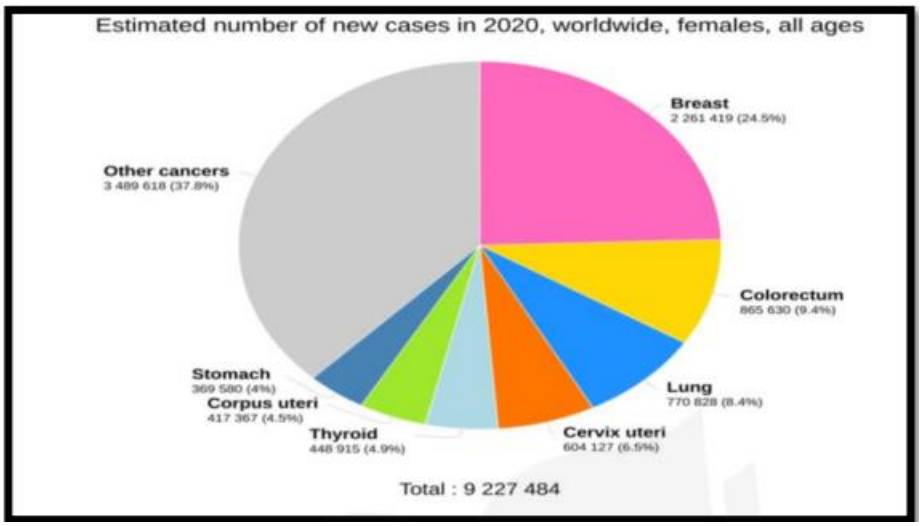
Figure 2-2: The structure of mammary gland lobule (Silverthon *et al.*, 2010).

When the tumor is small and treatable, breast cancer usually has no symptoms. The most frequent physical indicator is a painless lump or swelling . Breast soreness or heaviness, persistent changes in the skin, such as swelling, are less frequent signs and symptoms (Miyazaki *et al*., 2022).

2.4 Breast Cancer Incidence

Breast cancer is the second most prevalent disease in the world and the most frequent cancer in women (24.2 percent, or almost one in every four new cancer cases identified in women globally) (Giaquinto *et al.*, 2022) .

In 2020, an estimated 2.3 million new breast cancer cases were reported globally, accounting for 11.7% of all cancer cases in women and 685.000 (6.9%) breast cancer deaths, surpassing lung cancer as the most commonly diagnosed cancer (Sung *et al.*, 2021) .



(Figure 2-3):Rate of the incidence of female cancers in the world for the year 2020 (WHO,2020).

The highest rates are found in developed countries such as Australia and Western Europe, and the lowest in developing countries like Eastern Africa and the Middle East and South Asia (Bray *et al*., 2018).

A given geographical location, such as Africa, data on incidence and mortality remains exceedingly scarce (Hankinson e*t al*., 2020). According to the World Health Organization (WHO), breast cancer incidence rates are continuously growing in nations of the Eastern Mediterranean Region (EMR), including Iraq, with yearly increases ranging from 1% to 5% (Kulhánová *et al*., 2017) .

According to Iraqi research, the highest incidence rates of breast cancer are found in middle-aged women, with more than 40% of cases still being discovered at an advanced stage (Alwan and Kerr, 2018) .

In Iraq, breast cancer is considered the most common cancer (Iraqi Cancer Registry,2019).It ranks first among the commonest malignancies in all the population (Distribution of the top ten cancers. Iraq, 2019). (Alwan *et al*., 2022) .

There were 35864 cases in 2019 considered 6094 in females, the percentage of total constitute around 19.70 % with rate 16.3 for each 100000 population as reported in Distribution of the top ten cancers, Iraq 2019 (AL-saqabi , 2022).

While there were 9331 in Baghdad with rate 111.87/100.000.Baghdad population number 8340711as reported in Iraqi Cancer Registry-2019. (Annual Report Iraqi Cancer Registry , 2019).

Cancer is considered the main cause of illness and death globally and it is responsible for 19.3 million patients has been diagnosed and 10 million deaths on earth. The number of people with cancer increases to a 70% rate in countries that pay a low medium salary to their employees and workers.(Shkur , 2021).

2.5 Signs Symptoms of breast Cancer

There are many symptoms and signs that can coincide with breast cancer and the appearance of one .These symptoms or the following signs may indicate the onset of breast cancer, and it is not necessary for all of them to be present Symptoms in the occurrence of infection and these Symptoms:

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 (Sarker *et al*., 2022).

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, according to the report of the World Health Organization (WHO), 2003 .(Hussain *et al*., 2022)

2.6 Breast histological Grade

A histologic grade should be given to all invasive breast carcinomas (van Dooijeweert *et al.*, 2020).

Grade is a potent prognostic indicator that should be included in the minimal dataset for the histological reporting of breast cancer .as well as a crucial component of clinical decision-making tools and adjuvant internet resources (Rakha *et al*., 2023).

The Society of American Pathologists recommends and mandates the use of the Nottingham combined histologic grade (Nottingham variant of the Scarff Bloom Richardson (SBR) grading system) (Gandhi *et al*, 2023).

A tumor's grade is determined by assessing three morphologic features used for cytology grading (tubule formation, nuclear pleomorphism , and calibrated mitotic count).A combined score of 3-5 points is designated as grade I (low grade or well differentiation) and a combined score 6-7 points is grade II (Walke and Gunjkar , 2017).

2.6.1 Grade and Stages of breast cancer

The stage of the disease that is, the degree or spread of the cancer when it is initially diagnosed has a significant impact on the prognosis of invasive breast cancer (American Cancer Society, 2019a).

The TNM method is the most extensively used technique for staging breast cancer (Lester *et al*., 2012). Classification of the stages of tumors, and these classifications depend on 3 factors, which are (TNM) .

(T) indicates the size of the tumor, (N) indicates the size of the tumor in the lymph nodes and depends on the size of the glands.axillary lymph nodes, (M) stands for metastasis or the extent of tumor spread in other parts of the body .

The American Joint Committee on Cancer's (AJCC) TNM staging method for breast cancer applies to invasive and in situ carcinomas with or without microinvasion (Cabioglu *et al*., 2019) .

The stage is in situ if cancer cells are exclusively present in the layer of cells where they formed and have not disseminated, according to this concept. The cancer has become invasive when cancer cells have gone beyond the original layer of tissue, and it is classified as local, regional, or distant based on the level of dissemination (Liu *et al*., 2022).

The stages are:

1- breast cancer Stage 0

At this stage, the cancer is localized and has not spread to the breast and has not invaded neighboring cells. Removing it and keeping the breast or The lymph nodes at this stage do not contain any cancerous cells.

2- breast cancer stage I

It is an early stage of breast cancer, as it may affect neighboring tissues, and this stage means that the cancer has not exceeded the breast and that the size of the tumor at this stage is less than (2 cm) lymph nodes do not contain any cancer cells .

3- breast cancer Stage II

It is an early stage of breast cancer. Cancer spreads to the lymph nodes under the armpit, and that size . The tumor ranges between (2-5 cm) in

the lymph nodes, as they contain cancerous cells under the armpit, and the tumor . At this stage, it has not spread outside the breast.

4- breast cancer Stage III

Cancer spreads at this stage in abundance in the lymph nodes under the armpit, as the cancer is in these .Locally, the size of the tumor at this stage is larger (than 5 cm), and the lymph nodes contain the advanced stage.It contains cancerous cells under the armpit that are attached to each other, and the tumor is not spread outside the breast .At this stage, there is a node in the skin or skin ulcers in the breast, in addition to the presence of tumor nodes .Under the muscles or in the armpit area .

5-breast Cancer Stage IV

At this stage , cancer has spread from the breast to distant organs such as the bones , lungs , liver،The brain (Wang *et al.,* 2019) .

2.6.2 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 (Qiao *et al.,* 2022).

The grade is much more important for some kinds of cancer than for others. For most kinds, it is a somewhat secondary factor, but for a few kinds of cancer, 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 grade will also be found on the pathology report from your biopsy or surgery. For information on understanding pathology reports (Fox *et al.*, 2022).

The grades of breast cancer are divided into:

1- Grade 1 (grade-low).In this degree, cancer cells are very similar to normal cells and grow slowly . The chances of it spreading are low

2- Grade 2 (moderate- or intermediate-grade) . In this degree, cancer cells appear abnormal and their growth is faster than in the first degree

3- Grade 3 (high grade) .At this point, cancer cells show significant changes from normal cells and tend to grow in a different way faster and more likely to spread.(Kim *et al*., 2022 a).

2.7 Pathology of breast cancer

According to the pathogenesis of breast cancer, the disease is divided into 3 stages, as these stages reflect the extent of breast cancer depending.The spread of cancer in the body and the stages are:

1-The early stage

In its early stages, breast cancer is confined to the ducts that carry milk to the nipple.During feeding (Feeding Breast) to the lobules (this type of cancer is called Alfonso) Noninvasive cancer or in situ carcinoma .The majority of non-metastatic malignant breast cancers are ductal (Akram *et al*., 2022)

2 -The invasive stage

Breast cancer at this stage is diagnosed by its spread behind the ducts or lobules (Lobules) and to the peripheral areas of the breast tissue, as the

size of the tumor is an important sign of metastasis . Breast cancer, and metastatic malignant cancer constitutes 79% (Xue *et al*., 2022) .

3- The stage of metastasis

Cancer at this stage has spread to other areas of the body, such as nearby lymph nodes . Lymph nodes, and the most common areas for metastasis are the bone, the liver, the lung.and the Brain (Luo *et al*., 2022a).

2.8 Diagnosis of breast cancer

2.8.1 Breast Self-examination (BSE).

It is also known as self-awareness, in which a woman naturally examines her breasts and notices changes in the breasts .The importance of this examination is in the early detection of breast cancer and thus reduce the incidence of mortality, and it is also associated with Some realistic factors directly related to self-examination such as age, level of education and standard of living (Younis *et al.*, 2022).

2.8.2 Mammography examination of the breast

Mammography has the ability to detect calcifications and lumps in the breast . Mammography is one of the accepted tests for most women with breast cancer, and it is very sensitive examination rate (95.77%) . Treatment of breast cancer can be effective in its early stages, when it appears . Symptoms and pathological signs, as this examination works to detect whether there are cancerous tumors or not .And thus reduces mortality rates (Dadsetan *et al*., 2022).

2.8.3 Breast ultrasound or Ultra songography

These x-rays are used to see if the lump in the breast is solid or contains fluid Ultrasound is used in this examination (Mubuuke *et al*., 2023).

2.8.4 Breast Magnetic Resonance Imaging (MRI)

This examination is a complement to a mammogram, which is used to detect a tumor cancer in the lymph nodes under the armpit, which shows more details of the dense breast tissue, and this examination is not certified in early detection of breast cancer in general (Freitas *et al*., 2022).

2.8.5 Fine Needle Aspiration (FNA).

This examination is performed using a fine needle with the possibility of direct observation of the direction of the needle by imaging. Ultrasound in the case of non-obvious or deep tumors where fluid is withdrawn or a little bit of mass tissue by this needle to facilitate the examination of the sample in the laboratory (El chamieh *et al*., 2022).

2.8.6 Genetic tests

Recently, there has been a growing interest in breast cancer using molecular tests to help diagnose breast cancer. Early prediction of this disease. Most recent studies confirmed the existence of a positive relationship between increased frequency of mutations in the *BRCA1, BRCA2* genes, and the incidence of breast cancer.

There are many tests to detect mutations in the *BRCA1,BRCA2* gene (Bychkovsky *et al*., 2022). The most common of these methods is to search for changes in the genomic DNA (*BRCA1, BRCA2*), and changes in the protein produced by these genes, and such tests are carried out on a blood sample.

Several tests are carried out in the laboratory, and studies have indicated that genetic tests can detect cancer (De Silva *et al*., 2023).

2.9 Treatment

There are a number of methods used in the treatment of breast cancer, depending on its type and stage. These methods:

2.9.1 Radio therapy

In this type of treatment, high-energy rays are used to destroy cancerous tumor cells .The type of rays used in this type of treatment is the same type used in regular rays, but In higher doses, this type of treatment is used as a complement to surgical treatment in the case of the removal of a malignant tumor, as well as It is also used when surgical intervention cannot reach the tumor due to the presence of the tumor in areas. It is not recommended to remove the tumor from it, and this treatment is also used in cases where breast cancer has spread. (Medeiros Torres *et al*., 2022).

2.9.2 Chemotherapy

The goal of using chemotherapy is to reach as many areas of the body, as the substance can. Chemical are used to all cells of the body through the blood to eliminate cancer cells, and in most cases. Cases: Breast cancer is treated with a group of drugs that are given either by mouth or by intravenous injection intramuscular, growth chemotherapy treatments used to reduce the risk of breast cancer (Doxorubicin, Cytoxan, Fluorouracil) , (Whittaker *et al*., 2022) .

2.9.3 Hormonal therapy

Breast cancer tumors have nothing to do with female hormones, especially estrogen, because of this. The hormone makes some types of

cancer cells in the breast grow if the cancerous tumor contains a large number of estrogen and progesterone receptors, as well as hormone therapy or what prevents its secretion or Its effect on the breast One of the first methods used in the treatment of breast tumors is that this treatment has a role for women.

Those who have receptors for hormones in their tumors, especially (estrogen and progesterone receptors if (70%) of breast cancer cases are positive-receptor ER, as well There are many hormonal therapies that have been used to treat metastatic breast cancer such as (Toremifen , Megasterol , Tamoxifen , and Megasterol) , (Alwan *et al.*, 2022).

2.9.4 Immunotherapy

This manufactured treatment depends on the way the immune system works, especially with regard to the way in which it is treated. It distinguishes foreign cells from normal cells of the body and treats them as hostile cells and then destroys them.Treatments include the use of monoclonal antibody therapy, that the goal of using immunotherapy is to eliminate cancer cells for long periods and from without adverse effects on normal tissues, Trastuzumab is an effective immunotherapy for primary and metastatic breast cancer diseases (Debien *et al*., 2023).

2.9.5 Surgical Treatment

Surgery is one of the main pillars in the treatment of breast cancer, and it is one of the most common surgical methods in breast cancer. The treatment is parietal mastectomy (Mastectomy), as the aim of surgery is to remove tumors tissue as well as lymph node removal (Kunkler *et al*., 2023).

2.10 Risk factor in breast cancer:

Risk factors that increase the chances of developing this disease can be identified when they are present in people.

2.10.1 Aging

The strongest risk factor for breast cancer is the older woman ,The incidence of breast cancer increases with age, doubling about every 10 years until the menopause, when the rate of increase slows dramatically **(** Nguyen *et al*.,2023)

2.10.2 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 (Miller *et al*., 2023).

A family history of breast cancer does affect one's risk of 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 (Michaels *et al*., 2023).

Therefore, the important features in a family history that seem 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) (Rooney et al., 2023). The risk is 1.5 to 3.0 times higher if a mother or sister has the disease (Han *et al*., 2022) .

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 (Clements *et al*., 2022) .

In small groups of families, the patterns of breast cancer seem to be consistent with the known patterns of genetic inheritance (Zang *et al*., 2022).

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 (Dembrower , 2022).

2.10.3 Menstrual and reproductive history:

Breast cancer risk increases with early menarche and late menopause, and is reduced by the first full-term pregnancy (Tuo *et al*., 2022) .

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 (Manoucherhri *et al*, 2022) 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 (Coelingh *et al.*, 2023).

2. 10.4 The personal history of the injury

The presence of previous cancer in one of the breasts increases the incidence of breast cancer, especially if it occurs before menopause, ovarian or uterine cancer, and women with benign breast cancer. They have a high risk of breast cancer and within (4-5 times) (Mann *et al*., 2022).

2.10.5. 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 (Kim *et al*., 2022b).

2.10.6 Environment Factor

Environmental factors are believed to explain a large proportion of breast cancer incidence. Known risk factors for breast cancer, which are related to the reproductive life of women and other factors, such as inheritance and socioeconomic status, explain only about half of the breast cancer cases in the USA.(Terry, *et al.*,2019).

Ionizing radiation is a well-established environmental risk factor for breast cancer. (Jabbari, *et al*., 2019).

Chemicals that induce mammary cancer in rodents have served as leads for studies in humans, but occupational and environmental exposure to

these chemicals have for the most part lacked association with breast cancer risk. (Koual, *et al*. 2020).

However, recent evidence suggests that cadmium at very low doses acts as an estrogen mimic, indicating a need to investigate the effects of metals on breast cancer risk. Studies suggest that circadian rhythm disruption is linked with breast cancer, but too few studies have been done to be conclusive.

Over the years, cigarette smoking as a risk factor for breast cancer has remained controversial. However, recent research has found passive smoke exposure to be associated with increased breast cancer risk, which is hypothesized to be accounted for on the basis of an anti-estrogenic effect of smoking. Solar radiation has been noted to be associated with reduced breast cancer, supporting the hypothesis that vitamin D plays a protective role in reducing this risk (Crocetto, *et al* 2023).

Although, most of the environmental factors discussed in this review have not been convincingly found to influence breast cancer risk, research suggests that environmental exposure in combination with genetic pre-disposition, age at exposure, and hormonal milieu has a cumulative effect on breast cancer risk (Arthur *et al*., 2020).

2.10.7 Genetic Factors

Genetic factors represent (5-10%) of all breast cancer cases, especially if they are represented by the mother or One of the sisters, and that the risk of breast cancer is higher in women who have close relatives than First degree, mother (sister, daughter, father) infected with this disease, where the percentage rises to double, if either Second-degree

relatives (grandmother, aunt, aunt), whether from the mother or father, the infection rate rises, but be less than the first case (Lee *et al*., 2019).

The genetic causes are one of the most important causes leading to the occurrence of breast cancer, and include three important genes, *BRCA1*,*BRCA2*, *FOXP3*.

2.10.7.1. *BRCA1* gene (mutation):

BRCA1 a gene on chromosome 17 that normally helps to suppress cell growth. A person who inherits certain mutations (changes) in a *BRCA1* gene has a higher risk of getting breast, ovarian, prostate, and other types of cancer (Collins & Issacs, 2020) .

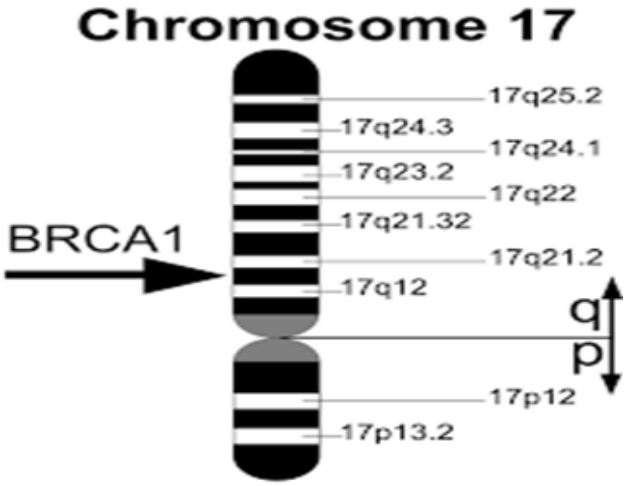
The function of the *BRCA1* gene is to prevent cancer. It is called the tumor suppressor gene. When working properly, tumor suppressor genes help prevent cancer by controlling cell growth and division (Bhin *et al.*, 2023).

It was mapped in 1990 to chromosome 17q21 by genetic linkage analysis of large families that included many cases of earlyonset breast carcinoma (Torrance, 2022).

BRCA1 is a complex gene made 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 that are thought to be involved in DNA- binding or in protein- protein interactions (Murciano-gorff *et al*., 2022).

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 (Comeaux

et al.,2022) Mutation carriers were 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 (Guzinam *et al*., 2022).



Figure(2-4):BRCA1 gene

The discovery of breast cancer genes has led to an explosive growth in cancer screening for population at risk (Devico *et al*., 2022).

Despite the genetic heterogeneity of breast cancer and the high prevalence of sporadic disease, several breast cancer susceptibility loci have been identified (Ow *et al*., 2019).

BRCA1 carriers will develop breast cancer in a percentage of 25 to 35 by the age of 70 (Yedjou *et al*., 2019).

2.10.7.2 *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 one 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

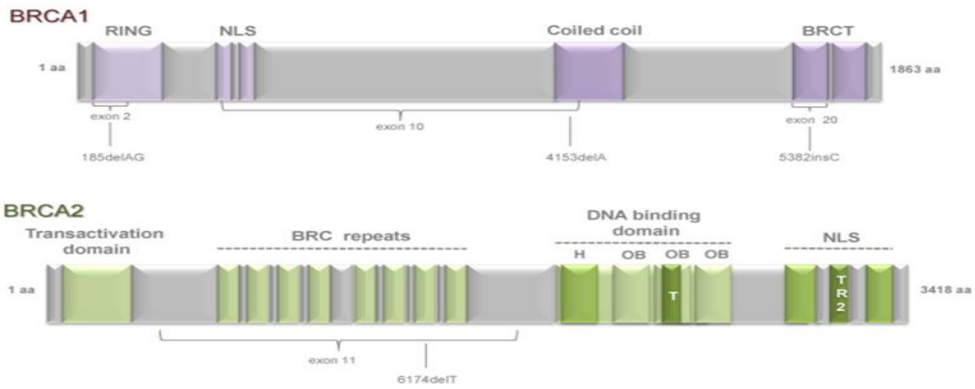
13q12 (Hossain *et al*., 2022) and subsequently cloned (Velaga *et al*., 2023).

BRCA2 is composed of 27 exons and encodes a protein of 3418 amino acid residues which does not appear to be significantly similar to other proteins (Lavoro *et al*., 2022).

It is clear that *BRCA2* is an important component of the pathway that protects cells from the effects of DNA damage (Van Den Tempel *et al*., 2019).

Recent studies have 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 (Castilllo *et al*., 2022).

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 (Burachik *et al*., 2023).



(Figure 2-5). Schematic representation of functional domains within BRCA1 and BRCA2 proteins and the position of several founder mutations.

BRCA1 is composed of 23 exons and BRCA2 includes 27 exons. Both genes encode large proteins: BRCA1 consists of 1,863 amino acids and BRCA2 of 3,418 amino acids. BRCA1 has a highly conserved zinc-binding RING (really interesting new gene) finger domain which is located close to the N-terminus. At the C-terminus, two BRCT (BRCA1 C-terminal) domains are located.

The central part of BRCA1 consists two NLS (nuclear localization signals) and one coiled coil domain. BRCA2 contains eight copies of a 20–30 amino acid repeat, termed BRC repeats. At the amino-terminus, BRCA2 has a TAD (transcriptional activation domain) domain and at the carboxyl-terminus two NLS and one TR2 domain.

DNA-binding domain is located close to the C-terminal region and is composed of a conserved helical domain (H), three oligonucleotide binding (OB) folds and a tower domain (T). Domains are indicated by violet (BRCA1) and green (BRCA2) boxes.

Domain names are shown above. Exons are indicated by braces. Positions of founder mutations are indicated beneath. (Gorodetska, et al.,2019).

2.10.7.3 *FOXP3* gene:

Is called forkhead box P3 *(FoxP3*), also known as scurfin is a protein-coding gene located at human chromosome Xp11.23is specifically expressed in CD4+ CD25+ regulatory T cells (Tregs) and functions mainly as a key regulator for the development and function of Tregs (Moradi *et al* ,2019).

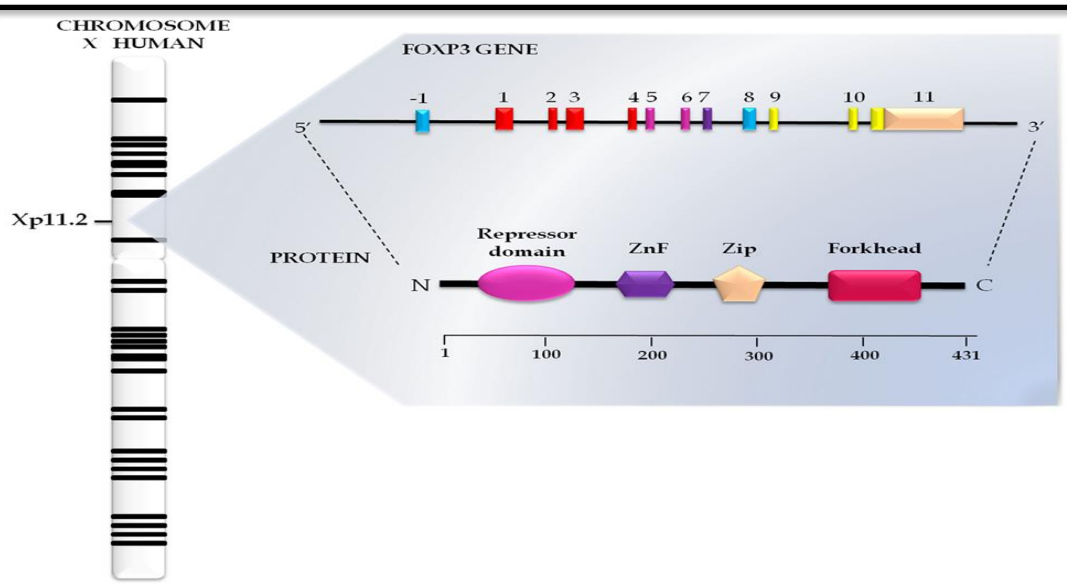


figure 2.6. genomics and structural organization of forkhead box protein 3 (foxp3).

The FOXP3 gene has 11 exons and is located on the short arm of the human X chromosome in Xp11.23 position. This gene encodes a protein FOXP3 has 431 amino acids structurally organized in the repressor domain, the N-terminal portion; zinc finger (ZnF) and leucine zipper (Zip), in the central portion; and forkhead domain, in the C terminal portion(Pereira *et al*, 2017).

As a member of the FOX family, FoxP3 also regulates transcription and DNA repair and is involved in cell growth and differentiation as well as embryogenesis(Jia *et al*., 2019). In addition to its critical function in immune response.

FOXP3 plays an important role in cancer development, although it is still a controversy whether it is an oncogene or tumor suppressor gene (Wang *et al*., 2023)

Forkhead Box Protein3 *(Foxp3),* a member of transcription factor winged-helix family is involved in regulating the immune system development and function . It was identified during positional cloning of

scurfin, a gene responsible for the X-linked autoimmune diseases in mice and humans (Jiang *et al*., 2021b).

Heterozygous mice for Foxp3 mutation develop spontaneous mammary cancer. Somatic mutations and chromosomal deletions are most frequently observed in breast cancer involving a minimal region of Foxp3 (Atashgaran *et al*., 2020).

Molecular studies have shown a down regulation of Foxp3 expression in the mammary cancer tissues compared with normal breast epithelial cells. Besides, Foxp3 inhibits the transcription of Human Epidermal Growth Factor Receptor 2(HER2/ErbB2), a major oncogene for breast cancer; it also down-regulates S phase kinase protein 2(Skp2), which plays an important role in cell cycle regulation thus inhibit tumor growth (Li, *et al*., 2019).

Further, it has been shown that Foxp3 expression is enhanced by sex hormones by influencing the proliferation of Treg cells (Kressler, *et al.*, 2021).

This may in turn alter disease susceptibility and tumor promotion/destruction. Several studies have been carried out dealing with polymorphisms of Foxp3 promoter region in various diseases including breast cancer in different populations (Chatrabnous *et al*., 2019).

Foxp3 is considered to be an X-linked tumor suppressor gene as it is known to suppress various types of cancers including breast cancer and several lines of evidence support this efficacy .(Gong, *et al*., 2020).

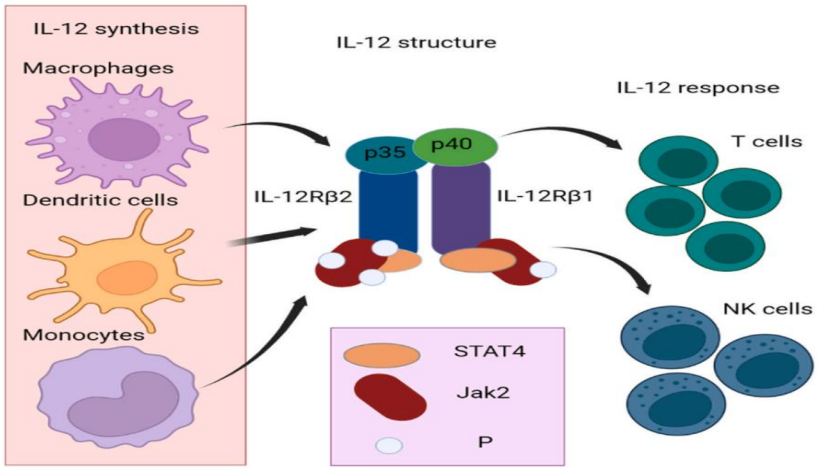
2.11 Inter leukien-12 (IL-12) .

Cancer is one of the most important issues nowadays, tremendous work has been done in past to diagnose and treat cancer. Many immunomodulation therapies are discovered especially the cytokines secretion for the treatment of cancer. Cytokines are small cell communication proteins.They coordinate by special signals in a paracrine manner. There are many families of cytokines in which the most diverse and important for immune responses is interleukins (ILs), one of which is IL12, an antitumor agent, which is very important in tumor immunotherapy (Yuzhalin ,2012).

interleukin 12 (IL-12) is an interleukin that is naturally produced by dendritic cells, macrophages, neutrophils, and human B-lymphoblastoid cells in response to antigenic stimulation. IL-12 belongs to the family of interleukin-12. IL-12 family is unique in comprising the only heterodimeric cytokines, which includes IL-12, IL-23, IL-27 and IL-35. Despite sharing many structural features and molecular partners, they mediate surprisingly diverse functional effects (Zhang *et al*.,2019) .

Interleukien-12 acts as a main mediator between the innate and adaptive immune systems, by controlling the proper development of naive CD4 T cells into diverse T helper (Th) subunits (Yan, *et al*, 2018).

Interleukien-12 is a heterodimer cytokine encoded by two separate genes, IL-12A (p35) and IL-12B (p40). The active heterodimer (referred to as 'p70'), and a homodimer of p40 are formed following protein synthesis. IL12A is composed of a bundle of four alpha helices. IL12B has three beta sheet domains.as shown in (Fig. 2-7).



(Fig.2-7)Structure of IL-12, the response cells and how are they form.

The expression of these two subunits is highly regulated. The p35 is produced by the expression of p40 for the secretion of the biological active cytokines and this process is limited, due to the abundant expression of p40 subunits, but the process is highly regulated and p40 is highly abandoned. The p40 binds covalently to the p35 allowing IL-12 to function (Gerber *et al*., 2021).

In the human genome, the subunit p35 is located on chromosome 3p12-3q13.2 and p40 is located on 5q31-33 (Bastian, *et al*.,2019).

The family of IL-12 contains 2 subunits alpha and beta. The alpha subunit is a four-helix structure with the receptor domain of haematopoietin containing p19, p28, and p35. The beta chain contains p40 and Ebi3 and is homologous with class I receptor chain cytokines (Floss *et al*., 2020).

Role of IL-12 in immunotherapy is an influential inflammatory cytokine that indicates a very interesting role in cancer treatments (Wang *et al*., 2017).

Immunotherapy based on cytokines has shown to be helpful in the treatment of a variety of cancers as IL-12 activates the cytolytic activity of NK cells and CD8+ T-lymphocytes which are responsible for tumor cell death (Mirlekar, *et al*.,2021).

In vivo, the antitumor impact of IL-12 in gene transfer contains numerous components from a molecular standpoint. After gene expression, IL-12 initiate tumor infiltran which include Th1 and natural killer cells action (killing tumor cells) at the site of the tumor. IL-12 also attracts and pilots the macrophages to the site of inflammation. Many studies have shown that the activity of IL-12 as an anti-tumor does not depend on the cytotoxic cellular responses but usually involves inhibition of angiogenesis which may be started in vivo by ultrasonography. No doubt IL-12 based gene therapy is effective in treating a variety of cancers (Chiocca, *et al*., 2019).

Role of IL-12 in cancer treatment has been proven to control both innate (NK cell) and adaptive (cytotoxic T lymphocytes) immunities. In many animal models, the antitumor impact of IL-12 is considered a powerful and evolving drug in cancer immunotherapy. Furthermore, the antitumor activity of IL-12 can be enhanced by its conjunction with many treatment modalities comprising antibodies, anti-angiogenic agents, radiotherapy, adaptive therapy, and antitumor vaccines (Lu, 2017).

Interleukien-12 is also capable of stimulating lymphocytes which decreases the growth of cancer cells (Tao *et al*., 2018) and increases T-cell proliferation

Various studies have shown an increase in immunosurveillance via IL-12 interference, such as the IL12 nanostimulant-engineered chimeric

antigen receptor T (CAR T) cell, which enhanced the antitumor immunity (Luo, *et al*., 2022b).

Interleukien-12 has been a key mediator of the immune response, by promoting T cells. Unlike other cytokines IL-12 is able to individually perform proliferation and cytotoxic functions, inducing antitumor cytokines (Shah *et al*., 2019).

Chapter three

Material and method

3.Materials and Methods

3.1 Subjects

3.1.1 Patients Study Group

The first group consists of 50 patient women diagnosed with breast cancer from the medical Merjan city in Babylon, The ages patients were range from 30-71 years with average age 51 years from different area of Babylon. Ethical permission to conduct the research was obtained from these hospital and from all participants in this study. Selections of the patients and the diagnosis were done under the consultant medical staff and pathologist committee at medical Merjan city in Babylon.

The diagnosis was made according to the clinical mamographic, histological findings, by which patients were early detected. Some of the patients received chemotherapy or radiotherapy or treatment with mastectomy Then blood samples were collected . The molecular detection was carried out by using conventional polymerase chain reaction (PCR) technique. The main data collected from patients in this study were: Age ,Family history, Ethnicity, Smoking ,Education level, Menstrual history and others.

3.1.2 Control Group

The second group a control group consisted of 50 subjects apparently healthy women , females with age range was from 30-70 years matched patients for ethnicity. All examined women were residents in a different areas of Babylon. The informed consent and agreement to give blood samples were obtained from all participants in this case – control study.

3.1.3 Samples Collection and Preparation

5 ml of peripheral blood were withdrawn from each patient and control , collected by disposable syringe, then blood samples divided into two parts: 2 ml in EDTA tube and in 1.5ml eppendorf tube for the molecular study. Blood with gel tube and eppendorftube were stored at -20ºC for further analysis.

3.2.Materials

3.2.1. Equipment

The equipment that used in the present study shown in table (3-1).

Table (3-1): The equipment which were used in the study

NO.	**Laboratory Equipment**	**Origin**
1.	Disposable syringe (5ml)	Easy med
2.	EDTA tube	High top
3.	Eppendrof tube	AFCO
4.	Gilson blue tips	AFCO
5.	Jell tube	High top
6.	Micropipette from100-1000 micro letter Micropipette from 0.1-3 micro letter Micropipette from 10-100 micro letter Micropipette from 0.5-10 micro letter	Eppendrof Research Plus(Germany) Biohit Nichiryo (Japan)
7.	PCR tube	Bionear
8.	PCR tube rack	Watson Bio Lab
9.	Spin column	Biocomma limited (spin)
10.	White Pipette tips	ExpellPLUS ™
11.	Yellow Pipette tips	AFCO
12.	Collection tube	Biocomma limited (spain)

3.2.2.Devices

The devices that used in the present study shown in table (3-2).

Table (3-2): The devices which were used in the study

NO.	Laboratory Devises	Manufacture company (Origin)
1	Autoclave	Hirayama
2	Eppendrof Centrifuge 5418	Eppendrof
3	Gel Electrophoresis devise	Advance
4	Gel Documentation	ATTA-E Graph (Japan)
5	Incubator	Memmert
6	Microplate reader	Molecular Devices, LLC
7	Microwave Oven	Shownic
8	Thermo cycler	Multigene
9	Refrigerator	Vestel
10	Sensitive balance	Kern (Germany)
11	Vortex mixer	Bioneer
12	Water bath	Memmert
13	ELISA reader	

3.2.3.Chemical and Biological materials

The Chemical and Biological materials that used in the present study shown in table (3-3).

Table (3-3): Chemical and Biological materials .

NO.	Materials	Manufacture company (Origin)
1	Agarose	Intron Biotechnology
2	Ladder 100-1000bp	Promega – USA
3	Ladder 50bp	Promega – USA
4	Loading dye	Intron Biotechnology
5	Nuclease-Free Water	Promega – USA
6	PCR Master Mix	Promega – USA
7	Primers (lyophilized)	Bioner-Korea
8	Red safe stain	Intron Biotechnology
9	Restriction enzymes	New England Biolabs
10	Tris Borate EDTA Buffer 10X (TBE)	Promega – USA

3.2.4. Kits and their contents

The kits that used in the present study shown in table (3-4).

Table (3-4): Kits and contents which were used in the study.

NO.	Kits	The industrial company	Contents
1	Whole Blood/ DNA extraction Kit.	FAVORGEN BIOTECH CORP.	▪ RBC lysis Buffer. ▪ FATG Buffer. ▪ FABG Buffer. ▪ W1 Buffer. ▪ Wash Buffer (Concentration). ▪ Elution Buffer. ▪ Proteinase K. ▪ FABG Mini Column. ▪ Collection tube. ▪ User manual.

3.2.4. Human Procalcitonin ELISA Kit (BT LAB).

2.3.4.1 Intended Use

This Sandwich kit is for the accurate quantitative detection of Human Procalcitonin (also known as PCT) in serum, plasma, cell culture supernatants, Ascites, tissue homogenates or other biological fluids.

2.3.4.2. Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human PCT antibody. PCT present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human PCT Antibody is added and binds to PCT in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated PCT antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human PCT. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

2.3.4.3.The contents

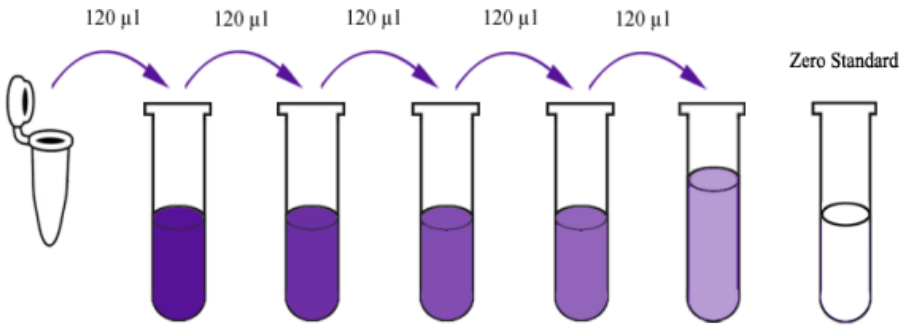
Standard solution (2400pg/ml), pre-coated ELISA plate, standard diluent, streptavidin-HRP, stop solution, substrate solution A, substrate solution B, wash buffer Concentrate (25x), biotinylated Human PCT antibody, user instruction and plate sealer.

2.3.4.4. Reagent Preparation

- All reagents were brought to room temperature before use.
- **Standard:** Reconstitute the 120ul of the standard (2400pg/ml) with 120ul of standard diluent to generate a 1200pg/ml standard stock solution. Allow the standard to sit for 15 mins with gentle agitation

prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (1200pg/ml) 1:2 with standard diluent to produce 600pg/ml, 300pg/ml, 150pg/ml and 75pg/ml solutions. Standard diluent serves as the zero standard (0ng/L). Any remaining solution should be frozen at -20°C and used within one month. Dilution of standard solutions suggested are as follows:

1200pg/ml	Standard No.5	120ul Original standard + 120ul Standard diluent
600pg/ml	Standard No.4	120ul Standard No.5 + 120ul Standard diluent
300pg/ml	Standard No.3	120ul Standard No.4 + 120ul Standard diluent
150pg/ml	Standard No.2	120ul Standard No.3 + 120ul Standard diluent
75pg/ml	Standard No.1	120ul Standard No.2 + 120ul Standard diluent



Standard concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
2400pg/ml	1200pg/ml	600pg/ml	300pg/ml	150pg/ml	75pg/ml

- **Wash Buffer:** Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If

crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

2.3.4.5. Assay Procedure

1. All reagents, standard solutions and samples were prepared as instructed. All reagents were brought to room temperature before use and the assay was performed at room temperature.
2. The number of strips required for the assay were determined. The strips were inserted in the frames for use. The unused strips were stored at 2-8°C.
3. 50µl of standard was added to standard well.
4. 40µl of sample was added to sample wells and then 10ul anti-PCT antibody was added to sample wells, then 50ul streptavidin-HRP was added to sample wells and standard wells (Not blank control well), and then were mixed well and covered the plate with a sealer and incubated for 60 minutes at 37°C.
5. The sealer was removed and the plate was washed 5 times with wash buffer. The wells were soaked with 300ul wash buffer for 30 seconds to 1 minute for each wash. The plate was blotted onto paper towels or other absorbent material.
6. 50µl of substrate solution A was added to each well and then 50ul substrate solution B was added to each well. The plate was incubated and covered with a new sealer for 10 minutes at 37°C in the dark.
7. 50µl of Stop Solution was added to each well, the blue color was changed into yellow immediately.
8. The optical density (OD value) of each well were determined immediately used a microplate reader set to 450 nm within 10 minutes after the stop solution was added.

3.2.4.2 Human level IL-12 assay by ELISA

Human IL-12 quantity was measured using the specific kit (ELISA) supplied by Elk - Biotechnology company, the standard recommended was according to the kit instruction 200,100, 50,25 ,12.5 ,6 .25 ,3.13 pg/ml as follows :

1. Sample addition: A 100µl of standard, blank, or sample per well was added. The blank well was combined with reference standard and sample diluent. Solutions were added to the bottom of micro ELISA plate well, avoid inside wall touching and foaming as possible. Then they were mixed gently and covered the plate with sealer. After that, the plate was incubated at 37 °C for 80 minute.

2. Biotinylated detection Ab: The liquid of each well was removed ,without washing. Immediately 100µl of Biotinylated detection Ab working solution was added to each well. Then it was covered with the plate sealer and gently taped the plate to ensure thorough mixing. The plate was incubated at 37 °C for 50 minutes.

3. Washing: Each well were aspirated and washed, the process was repeat with approximately 200µl wash buffer (a squirt bottle, multi-channel pipette, manifold dispenser or automated washer were needed). Subsequently, the washing buffer was completely removed at each step and it was essential. After the last washing step, the wash buffer was removed by aspirating or decanting and then the plate was inverted against a thick clean absorbent paper to remove any traces of the liquid.

4. HRP Conjugate addition: A 100µl of HRP conjugate working solution was added to each well and then the plate was covered with the plate sealer. The plate was incubated at 37 °C for 50 minutes.

5. Washing: the washing process was repeated for five times as mentioned in the step 3.

6. Substrate addition: A 90µl of substrate solution was added to each well. Subsequently, the plate was covered with a new plate sealer and incubated at 37 °C for about 30 min in dark to protect it from light. The reaction time can be shortened or extended according to the actual color change, but not more than 30 min. When the obvious gradient appeared well, the reaction was terminated.

7. Stopping reaction: A 50µl of stop solution was added to each well. Then, the color turns to yellow immediately.

8. Optical Density Measurement: The optical density (OD value) of each well was determined at 450 nm immediately using a micro-plate reader. rated three times. The washing was performed by filling each well.

Typical Human IL-12 (p70) ELISA Kit Standard Curve

(TMB reaction incubated at 37°C for 15 min)

Concentration (pg/ml)	0.0	7.8	15.6	31.3	62.5	125	250	500
O.D.	0.201	0.279	0.317	0.446	0.627	1.084	1.856	2.333

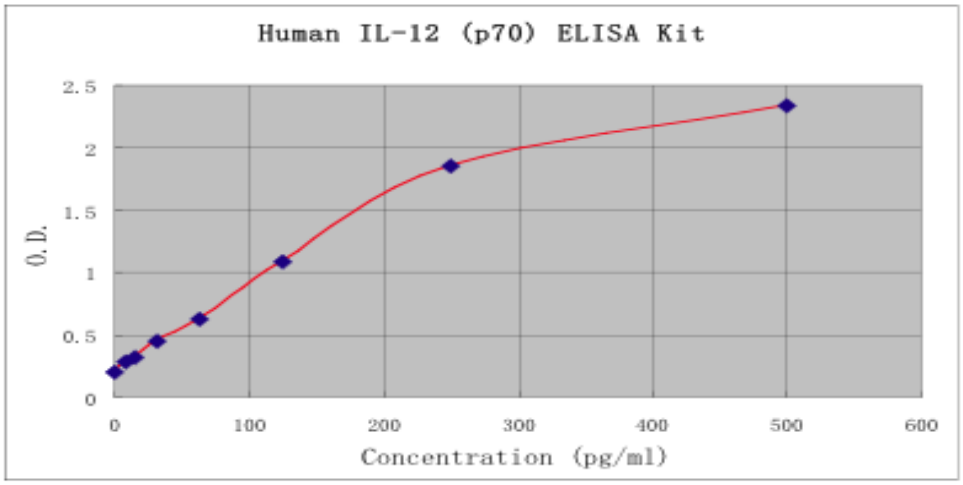


Figure (3-1) : Standard curve of Human IL-12 Assay concentration

3.2.5.Molecular materials

3.2.5.1.Primers

The primers that used in the present study shown in table (3-5).

Table (3-5) Shown the primers sequences and their size of amplicon used in the study

Primer	Sequences	Gene region & Location	Size Of Product (bp)	Cent.	Ref.
BRCA1 185del AG Gene	**P1** 5'GGTTGGCAGCAATATGTGAA '3 **P2** 5'GCTGACTTACCAGATGGGACTCTC '3 **P3**5'CCCAAATTAATACACTCTTGTCGTGA CTTACCAGATGGGACAGTA '3	Chr.17	335bp 354bp	59 C^0	Chan et al., 1999)
BRCA2 6174del T Gene	**P4** 5'AGCTGGTCTGAATGTTCGTTACT '3 **P5** 5'GTGGGATTTTTAGCACAGCTAGT '3 **P6**5'CAGTCTCATCTGCAAATACTTCAGGG ATTTTTAGCACAGCATGG'3	Chr.13	151bp 171bp	55 C^0	(Chan et al., 1999)
Foxp3-3279C/A (rs3761548) Gene	**P7** F:5'GCCCTTGTCTACTCCACGCCTCT-3' **P8** R:5'CAGCCTTCGCCAATACAGAGCC -3'	Chr.xp11.23	487bp	63 C^0	(Ebert et al.,2008.)

Promoter: P1,P4,P7=common reverse P2, P5=wild-type forward .P3, P6,P8 =mutant reverse

3.2.5.2.Restriction Enzyme

Restriction Enzyme that used in the present study was shown in table (3-6).

Table (3-6) The preparation mixture for *Pst I* Restriction Enzymes

Materials	Volume (µl)
PCR product	10
Enzyme	0.5
Buffer B	2
BSA buffer	0.2
Muti core buffer (10 X)	7 (1X)
DH_2O	16.2

The incubation time and temperature for Restriction Enzyme that used in the present study was shown in table (3-7).

Table (3-7): The incubation time and temperature for restriction enzyme (*Pst* 1). Providencin stuartti

Restriction Enzymes	Incubation time	Incubation temp.	The industrial company
Pst I(5' CTGCA$^{\downarrow}$G 3')	4 hours	37℃	Promega

Table (3-8) The Reaction Mixture for *Pst I* Restriction Enzymes .

Materials	Volume (µl)
PCR product	10
Enzyme	1
10X NEB buffer	5
DH_2O	34
Total volume	50

3.3.Methods

3.3.1.Design of the study

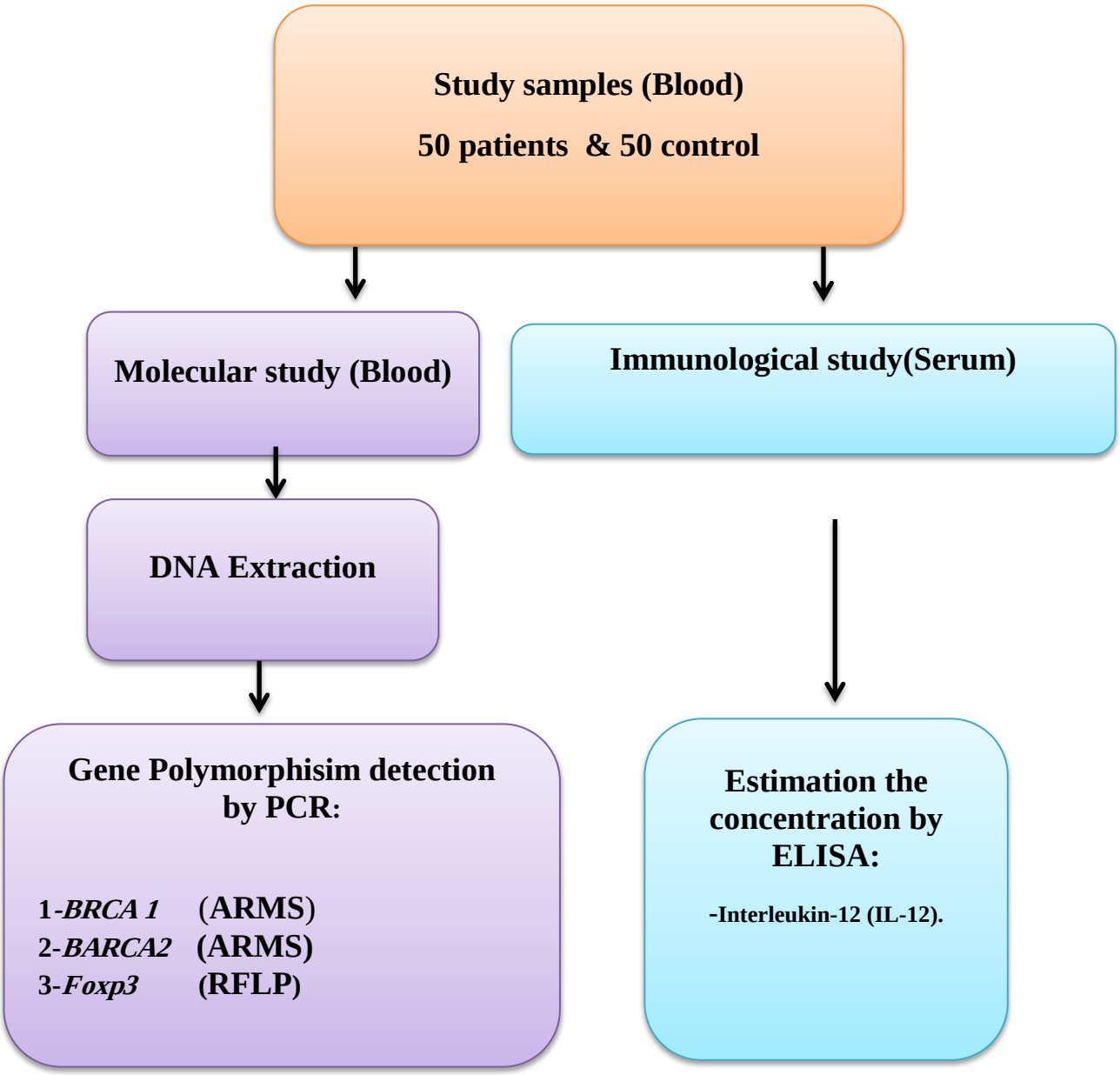


Figure (3-2) :Design of the study.

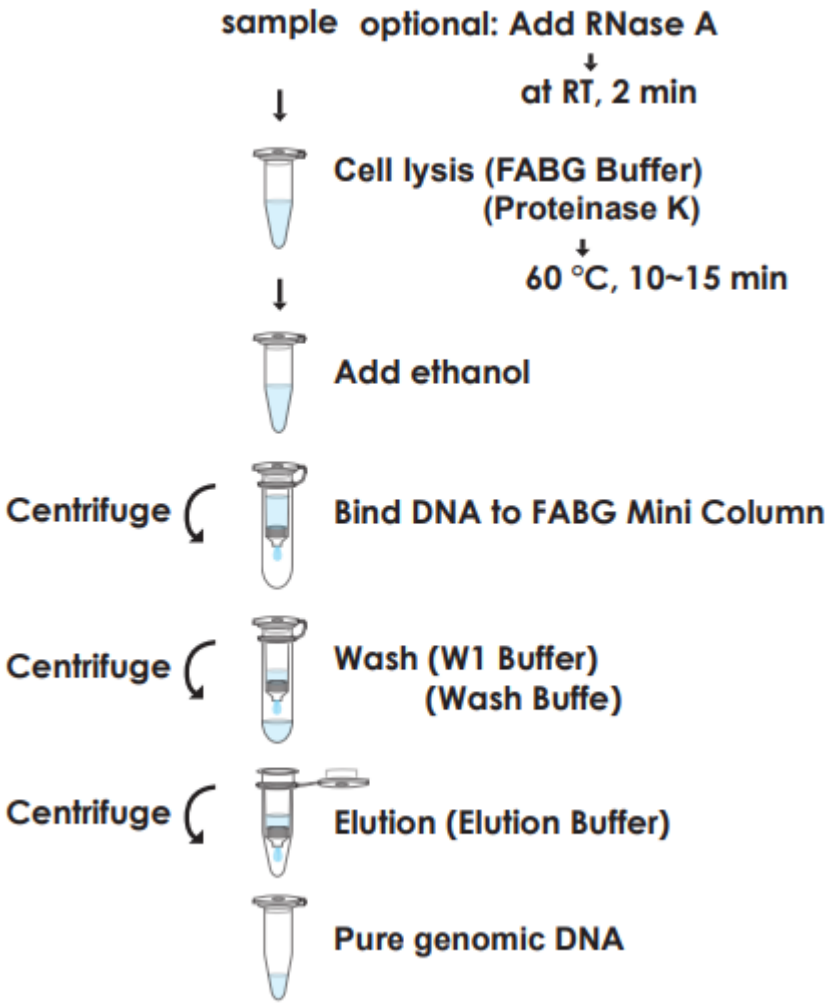
3.3.2.Blood DNA extraction Kit (Favorgen Biotech Corp).

This kit is used for extraction of genomic DNA from fresh blood, frozen blood, cultured cells and fungus.

3.3.2.1.Procedure

1. **Sample preparation:** Two hundred microliter of blood were transferred up to a 1.5ml microcentrifuge tube, 30 µl of proteinase K was added to the sample and was mixed briefly and was incubated for 15 min at 60 °C.
2. **Cell lysis:** Two hundred microliter of FABG buffer was added to the sample and was mixed by vortex and then was incubated in a 70 °C water bath for 15 min to lyse the sample and during incubation, the sample was inverted every 3 min.
3. **DNA binding:** Two hundred microliter of ethanol (96-100%) was added to the sample and was vortexed for 10 sec, the sample was pipetted to mixed well if there is any precipitate formed. FABG column was placed to a collection tube, the sample mixture was transferred to FABG column and was centrifuged at speed 14000 rpm for 1 min, and the collection tube was discarded and the FABG column was placed to a new collection tube.
4. **Column washing:** Four hundred microliter of W1 buffer added to the FABG column and was centrifuged for 30 sec at 14000 rpm and then the flow-through was discarded and the FABG was placed back to the collection tube. Then 600 µl of wash buffer was added to the FABG column and was centrifuged for 30 sec at speed 14000 rpm and then the flow-through was discarded and the FABG was placed back to the collection tube and then was centrifuged for an additional 3 min at speed at 14000 rpm to dry the column.

5. **Elution:** The dry FABG column was placed to a new 1.5 ml microcentrifuge tube, then 100 µl of preheated Elution buffer was added to the membrane center of FABG column, and then the FABG column was incubated at 37 °C for 10 min in an incubator, then was centrifuged for 1 min at full speed 14000 rpm to elute the DNA and then the DNA fragment was stored at -20 °C. The brief procedure shown in figure (3-1).



Figure(3-3): Brief Procedure for DNA Extraction.

3.3.3.Sterilization Methods

The instruments that were not affected by heat were sterilized in an autoclave at 121° C for 15 minutes at atmospheric pressure of 1.5 pounds per $inch^2$.

3.3.4.Tris Borate EDTA Buffer (TBE Buffer) preparation

Tris Borate EDTA Buffer was prepared by diluting 100 mL Tris Borate EDTA (10x) in 900 mL distilled water to make Tris Borate EDTA (1x), which was then used to prepare Agarose for the Gel electrophoresis.

3.3.5.Agarose preparation

For preparation of Agarose gel follow the steps :

1. One hundred ml of 1X TBE buffer was taken in flask.
2. Agarose powder (1.5, 2, and 3) was add to the 1x TBE buffer to prepare agarose gel in concentrations of 1.5%, 2% and 3% respectively.
3. The solution was heated to boiling using a microwave oven until all agarose particles were dissolved.
4. The solution was left to cool down.
5. About 5 µl of red safe was added to the agarose solution.

3.3.6.Conventional Polymerase Chain Reaction (PCR)

3.3.6.1.Primers Preparation

All primers used in this study were prepared for amplification studied genes, and that by dissolved the primers (Forward and Reverse) in 300 µl of Nuclease Free Water according to the supplied company instructions (Humanizing Genomics Macrogen) to obtained working solution. The tubes were shaken, then the final solution was prepared by

diluted 10 µl of primers (Forward and Reverse) in 90 µl of Nuclease Free Water (10 pmol/µl).

3.3.6.2.Polymerase Chain Reaction Mixture

The mixture of polymerase chain reaction was prepared to amplify the *BRCA1*,*BRCA2* and *Foxp3* genes by mixing the components master mix with the forward and reverse primers, the DNA template and Nuclease free water as shown in Table (3-9).

Table (3-9): Polymerase Chain Reaction Mixture

NO.	Materials	Volume	Concentration
1	DNA Template	6 µl	50 ng
2	Master Mix	12.5 µl	1X
3	Forward Primer	1 µl	10 pmol/ml
4	Reverse Primer	1 µl	10 pmol/ml
5	Nuclease-Free water	4.5 µl	
Total	Final Volume 25 µl		

A. *BRCA1* gene polymorphism

The PCR products of target regions *BRCA1* gene were electrophoresed on 2%agarose at 15 min on high (50 volt)&60 min on low an visualized by Red safe. Photos were taken using gel documentation system.

This polymorphism was detected by using amplification refractory mutation system (ARMS) PCR using two separate sets each containing the common revere primer and only one of the forward primers .The program that used to the amplification of the primer is shown in the table (3-10).

B. *BRCA2* gene polymorphism

The PCR products of target regions *BRCA-2* gene were electrophoresed on 2% agarose at 15 min on high (50 volt) and 60 min on low an visualized by Red safe . Photos were taken using gel documentation system.

This polymorphism was detected by using amplification refractory mutation system (ARMS) PCR using two separate sets each containing the common revere primer and only one of the forward primers . The program that used to the amplification of the primer is shown in the table (3-10).

C. *Foxp3*-3279 gene polymorphism

The PCR products of target regions *BRCA2* gene were electrophoresed on 2% agarose at 15 min on high (50 volt) and 60 min on low an visualized by Red safe .Photos were taken using gel documentation system.

After that, the amplified fragments of *Foxp3*-3279 gene , digested by *Pst I* Enzymes respectively to investigate restriction fragment length polymorphisms (PCR-RFLP).The program that used to the amplification of the primer is shown in the table (3-10).

3.3.6.3.Amplification Conditions

Polymerase Chain Reaction or thermo cycler was used to amplified the *BRCA1*,*BRCA2* and Foxp3 genes, the amplification conditions each gene have been controlled as shown in a table (3-10).

Table(3-10):Amplification Conditions for Polymerase Chain Reaction

Gene region	Initial denaturant .°C (min)	Denaturat. °C (min)	Annealing °C (min)	Extension °C (min)	Final extension °C (min)	Cycles	Ref.
BRCA1 **185del AG**	94ºC (5min)	94ºC (30s)	59 ºC (30s)	72ºC (30s)	72ºC (5min)	35	(Hassan,2019)
BRCA2 **6174del T**	94ºC (5min)	94ºC (30s)	55 ºC (30s)	72ºC (30s)	72ºC (5min)	30	(Hassan,2019)
Foxp3**-3279 C-A(rs3761548)**	94 (5min)	94 (30s)	50.5^{0}C (1min)	72^{0}C (1min.)	72^{0}C(5min)	30	(AL-Hajaj *et al*,2015)

3.3.6.4.Loading of DNA and Electrophoresis

The gene amplification products from the polymerase chain reaction (PCR) were loaded using the Agarose gel. The agarose was then fully dissolved and cooled at room temperature. The agarose was poured in casting tray that combs were fixed on it and then was lifted to harden at room temperature.

The gene amplification products (Amplicons) and loading dye were loaded into the pits that resulted after lifting the combs. The DNA ladder (5µl) was putted in a first and last pits in first line of pits and only in a first pit in second line for the purpose of comparing the molecular size of the resulting bands then relay tank was filled with a buffer (1X TBE) and the electrophoresis of *BRCA1&BRCA2* and *Foxp3* genes amplification products were performed with a voltage of 100 V for a duration of 45 minutes. Later the gel visualized using UV trans-illuminator and documented by digital camera (Hasan, 2019)

Chapter four

Result & discussion

4. Results and Discussion

4.1. Risk factor in Breast cancer

In this study the distributing of patients into age groups revealed 17cases of patients (34%) were at the age group (50-59) years old, 13 cases (26%) were at the age group (40-49)years old, 12 (24%) cases were at the age group (≥ 60) years old, 8cases (16%) were at the age group (30-39) years old ,Table (4-1).

Breast cancer women recorded in this study were divided according to menstruation into two subgroups, pre-menopausal and post-menopausal, the results showed that 68% of patients in pre-menopausal age and 32% of women were in post-menopausal age, significant differences found at$P \leq 0.001$, this results agreed with (Li *et al*., 2006 ; Saeed *et al*., 2020). Data in premenopausal women have been sparse, because of complexities in measuring estrogen levels during the menstrual cycle (Wang and Song , 2021).

There was a statistically significant in the distribution of frequencies in Family history of breast cancer and controls ($P<0.001$, OR 18.39 ,95% CI (5.04 to 67.00).

The higher incidence of breast cancer in family history is related to many factors that have collectively a cumulative effects including reproductive factors, genetic factors, hormonal factors, exposure to radiation and life style. (Burguin *et al*., 2021)

Table (4- 1) Distribution of the study sample according to Age, Marital status , Menopausal & Family History of Breast cancer.

Variable	Cancer Patients N=50 %		Control N=50 %		OR(95%CI)	P-value
Age Group	**N.**	**%**	**N.**	**%**		
30-39 [a]	8	(16%)	25	(50%)	0.17 (0.05 to 0.58)	0.004
40-49	13	(26%)	7	(14%)		
50-59	17	(34)	8	(16%)	0.15 (0.04 to 0.47)	0.001*
Above 60	12	(24%)	10	(20%)	0.26 (0.08 to 0.84)	0.002
Total	**50**	**100 %**	**50**	**100 %**		
Marital status						
Unmarried	1	(2%)	15	(30%)	0.04 (0.01 to 0.20)	0.003
Married	49	(98%)	35	(70%)		
Total	**50**	**100**	**50**	**100%**		
Menopause						
Postmenopausal	16	32%	37	(74%)	0.16 (0.07 to 0.34)	0.001*
Premenopausal	34	68%	13	(26%)		
Total	**50**	**100%**	**50**	**100%**		
Family History						
Positive	27	(54%)	3	(6%)	18.39 (5.04 to 67.005)	0.001*
Negative	23	(46%)	47	(94%)		
Total	**50**	**100**	**50**	**100%**		

P<0.001: OR =(95%Cl): [a] Reference

4.2.1 Aging

Results presented in this work had shown that women (50 cases of breast cancer) that most patients 17cases (34%) were in the age group (50-59) years old, 13 cases (26%) were in the age group (40-49) years old, 12 (24%) cases were at the age group (≥ 60) years old, 8cases (16%) were at the age group (30-39)years old, as presented in Figure 4.1

The results of present study were in disagreement with Jiang *et al*., 2021, who demonstrated that about 35–45% of Eastern breast cancer women aged less than 40 years. So that several observations which line of with present study that breast cancer in young women behave differently compared with breast cancer in middle-aged and elderly women.

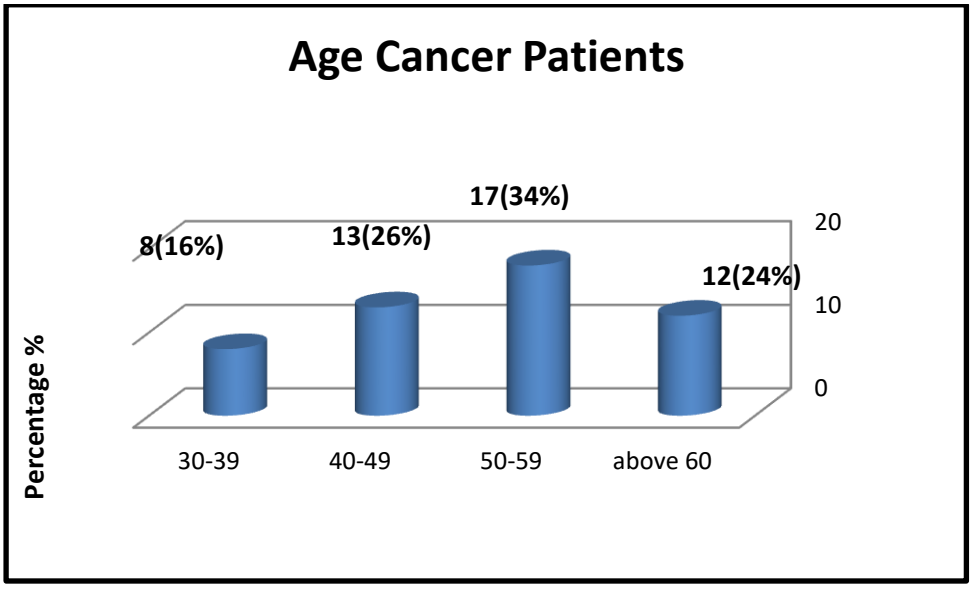


Figure 4.1:Distribution of the patients infected with breast cancer according to the age.

Breast cancer risk is strongly related to age and it is the most commonly diagnosed cancer in women above 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 1Thormonal 1T 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 is often 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 (Ozmen et al., 2009).

While (Alpaugh & 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 occurs in a small percentage of 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 of 35 and 75 years. Breast cancer in men is almost always seen beyond the age of 65 years.

4.2.2 Marital status

Results indicated in table (4-1) ,Figure 4 shown that 49 (98%) patients were already married while the other patients 1 (2%) are unmarried, as presented in Figure 4.2.

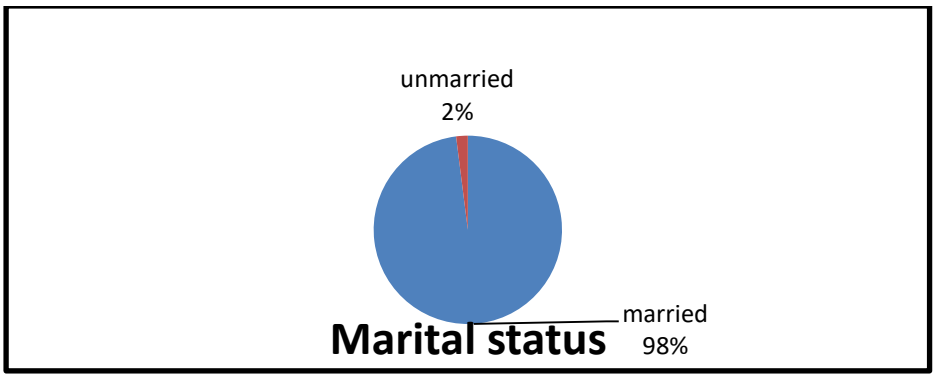


Figure 4-2: Distribution of Marital status among breast cancer cases.

Marital status was not a risk factor for breast cancer according to other studies that show that unmarried women are at higher risk for breast cancer (Huang *et al*., 2021).

In most studies single and nulliparous married women were found to have a similar increased risk for breast cancer as compared with parous women of the same age (Wang *et al*., 2019). Thus, one may argue that marital status by itself is not a determining factor for increased or reduced breast cancer risk and rather the main protective effect is from an early first full-term pregnancy.

In the present study no association with parity. Evidence suggests that there is an interaction between marital status and parity (Alshareef *et al*., 2020 ,Duffy *et al*., 2017) , supporting a dual effect of parity on breast cancer risk with pregnancy.

4.2.3 Menopause

Results indicated in table (4-1) shown that 16(32%) are postmenopausal, while the other patients 34 (68%) have premenopausal. This may be due to effect of aging as the menopausal women are already old, or to the high postmenopausal blood estrogen levels which is established as risk factors (Mercogliano *et al.*, 2022 ; Chlebowski *et al.*, 2020) .

AL-Bedairy *et al.*,2020 in Iraq documented an increased serum estradiol and prolactin among the pre- and post-menopausal breast cancer women, and recommends emphasizing the necessity of co-operation between the Ministry of Health and the Ministry of Commerce and protecting them from dangerous behavior by providing them with sufficient support and guidance to stay away from the hormonic products

and focusing on the extension programs in the protection of the community through educating them with the guidance

Late menopause increases the risk of breast cancer. Risk increases by almost 3% for each year older at menopause, so that a women who has the menopause at 55 years rather than 45 years, has approximately 30% higher risk. (Zobair *et al.*, 2020)

4.2.4 Family history

Family history of breast cancer indicates some association with risk of developing breast cancer. The present study showed that family history positive in 50 cases (54%) and (46%), had negative family history of breast cancer, as presented in Figure (4-3).

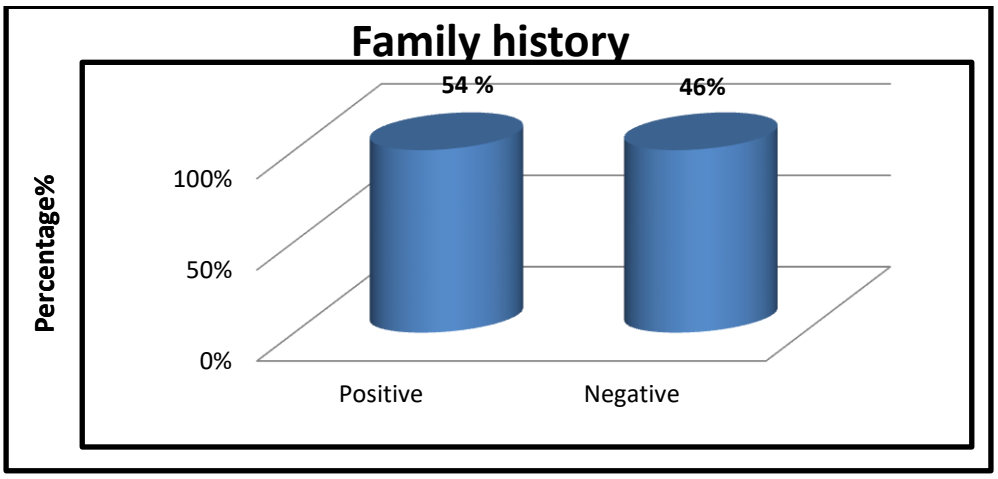


Figure 4-3: Distribution of Family history among breast cancer cases

Breast cancer, as a heterogeneous disease, is susceptible to genetic, hormonal and environmental risk factors that reflect a variety of characteristics which correlate with its prognosis. Women with a strong family history of breast cancer could inherit genetic alterations that modify their risk and clinical presentations. Accordingly, the second part

of this study was designed to evaluate the clinico-pathological characteristics of patients with positive family history of breast cancer**.**

Family history of cancer is risk factors for breast cancer. This is in accordance with other research findings indicating that a positive family history of breast cancer is a strong risk factor for breast cancer at young age (Turkoz *et al*., 2012) although this has a comparatively small effect on the absolute lifetime incidence of and mortality from breast cancer (Jacobi *et al*., 2003 ; Laforest *et al*., 2021).

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 (Dembrower , 2022).

4.3 Immunological factor in Breast cancer

The immunological parameters have been increased in response to Breast cancer infection (IL-12). This result might be refer to the fact that the immune system plays as protective role and has prognostic indicator for disease, activity and past infection (immunization). These result are shown in Table (4-2).

Table (4-2) : Age distribution in relation to IL12 levels in patients and control.

Age and Immune Markers		Control Mean ± SD	Breast cancer Mean ± SD	P-Value
IL12(Pg/ml)	30-39 year	41.7906 ±5.2747	22.1938±5.1858	1.2
	40-49 year	38.4186±7.212978	33.007 ±5.7589	0.02*
	50-59 year	45.95349±7.365456	29.91473±7.649229	1.3
	Over 60	44.44961±4.299853	34.66408±5.77202	0.02*

T-test at P ≤ 0.005

(Mean ± SD): Mean± Standard Deviation .

The current study results investigated whether immune system impairment manifested by reduced IL-12 levels ensue in BC patients. The present study demonstrated evidence indicating that IL-12 was decreased in BC patient's serum, suggested that tumor derived IL-12 was associated with tumor progression .These results were in agreement with (Rao *et al*, 2004) who demonstrated significant differences in levels of serum IL-12 in patients as compared with controls and revealed that the IL-12 level was not correlated with stages of BC disease. (Rao *et al.*, 2008)

These findings are compatible with a study finding that indicated the reduced IL-12 production may contribute to the tumor formation and development (Mercogliano *et al.*, 2022).

Cytokines play varied roles in cancer pathogenesis, with increasing evidence suggesting their involvement in tumor initiation, growth and metastasis (Kovacs,2001). IL-12 is a proinflammatory cytokine. The potent anti-tumor activity of IL-12 has been demonstrated in many preclinical murine tumor models (Al-Ghurabi, 2009).

Interleukien-12 which is produced primarily by monocytes/macrophages and B-cells, is an important cytokine in the activation of cell-mediated immunity. IL-12 has potent antitumor activity, and is used in the immunotherapy of cancer. It appears that patients with elevated blood concentrations of IL-12 have a higher survival rate than patients with low concentrations (Yan *et al.*, 2017).

4.4 Genetic Factor in Breast cancer

4.4-1 Genetic polymorphisms of some related-genes associated with Breast cancer

The genomic DNA in figure (4-4) was extracted from the blood samples as a first step to amplify the target region of *BRCA1*, *BRCA2* and *FOXP3* gene.

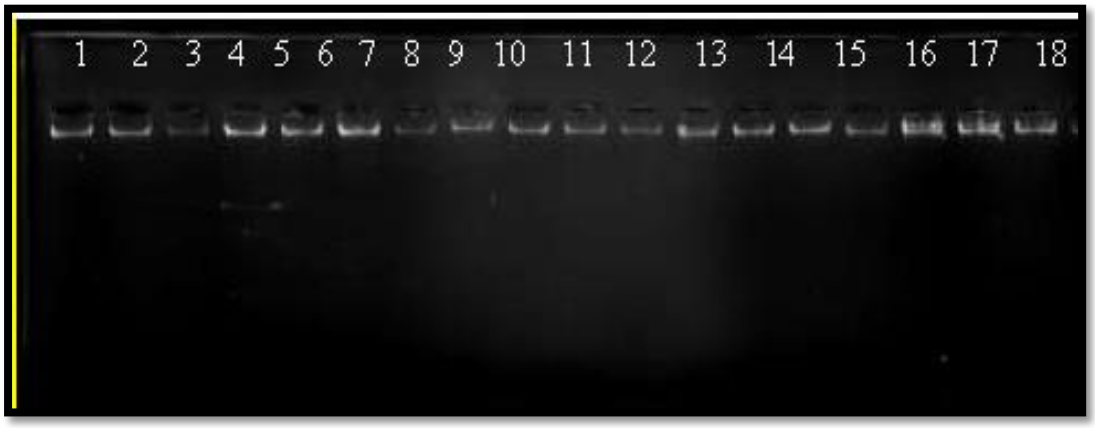


Figure 4-4 : Electrophoresis pattern of genomic DNA extracted from blood samples of patients and healthy control groups. Lane 1 refers to genomic DNA from blood samples (1-10 patients and 11-18 control) ; Electrophoresis conditions, 1% agarose, red safe stained 5 Ml for 15 min on high (50 volt) and 60 min on low.

4.4. 2 185delAG *BRCA1* gene Polymorphism

Table (4-3) Shows the distribution of 185delAG mutation of *BARCA1* gene in control and patients Breast cancer. AA 52%, AG 32%

and GG 16% in controls and 18%, AG 46% and GG 36% in cases. An increased frequency of homozygote mutant genotypes (GG) was found in patients compared to controls. There was a statistically significant difference in the distribution of allele frequencies in cases and controls (AG: P<0.005, OR 0.32 , 95% CI 0.18 to 0.58) .

The mutant 185del AG was originally detected with a high frequency in Ashkenazi Jews (Friedman *et al*., 1995). This result was agreed with (Tikhomirova *et al*., 2005), who also did detect the 185delAG mutation in 55 Chilean women affected by breast cancer, 15 of whom had a positive family history and 40 with sporadic breast cancer. In the last few years many studies have focused on screening of mutations in breast cancer.

Table (4-3): Genotype and allele distribution 185delAG *BRCA1* gene polymorphism in patient and control, shown the Odd Ratio value.

Genotypes	Patients (N=50)		Control (N=50)	OR(95%CI)	P-value
BRCA1	A/A[a], n(%)	9(18%)	26 (52%)		
	A/G, n(%)	23(46%)	16 (32%)	0.24 (0.08 - 0.64)	0.004*
	G/G, n(%)	18 (36%)	8 (16%)	0.15 (0.05 - 0.47)	0.001 *
Allele Frequency					
	A, n (%)	41 (0.41)	68 (0.68)	0.32 (0.18 - 0.58)	0.001*
	G, n (%)	59 (0.59)	32 (0.32)		

AA-homozygous wild , AG-heterozygous, GG-homozygous mutant

P<0.005: OR =(95%Cl): [a] Reference

Figure (4) shows the samples AG (1,3,7,6,8and 9) are heterozygous for this type of mutation, first lanes represent the sample; lanes 4,5 WT (wild type specific amplification, second lanes 335bp fragment and 2MT (mutant-specific amplification 354bp fragment .

Figure(4-5):: Represents amplification Products of *BRCA1* ; A: wild-type alleles with 335bp by wild-type specificprimers (4,5) with DNA samples in Group B: Mutant-type alleles with 354bp by mutant -type specificprimers (2) with DNA samples in Group A. WM-heterozygous(1,3 6,7,8and 9) On a 2% agarose gel, red safe stained 5 Ml for 15 min on high (50 volt)&60 min on low. There was a DNA molecular marker in Lane M.(100 bp).

4.4. 3 *BRCA2* gene Polymorphism

Table (4-4) shows the distribution of *BRCA2* gene in control and patients Breast cancer. TT 46%, TA 34% and AA 20% in controls and 22%, TA 42% and AA 36% in cases. An increased frequency of

homozygotic mutant genotypes (AA) were found in patients compared to controls. There was a statistically significant difference in the distribution of allele frequencies in cases and controls (TA: P<0.005, OR 0.44, 95% CI (0.25 to 0.78) .

A large number of distinct mutations in the BRCA2 genes have been reported worldwide, also known regarding the role of this susceptibility gene as risk factor for breast cancer in Iraq. The BRCA2 gene mutations may vary according to the characteristics of the patient groups as well as the ethnic and genetic features of the screened population.

Table (4-4): Genotype and allele distribution *BRCA2* gene polymorphism in patient and control, shown the Odd Ratio value.

Genotypes	Patients (N=50)		Control (N=50)	OR(95%CI)	P-value
BRCA2	T/T[a], n(%)	11 (22%)	23 (46%)		
	T/A, n(%)	21(42%)	17 (34%)	0.38 (0.14 – 1.01))	0.051
	A/A, n(%)	18 (36%)	10 (20%)	0.26 (0.09 - 0.76)	0.012*
Allele Frequency	T, n (%)	43 (0.43)	63 (0.63)	0.44 (0.25 - 0.78)	0.005 *
	A, n (%)	57 (0.57)	37 (0.37)		

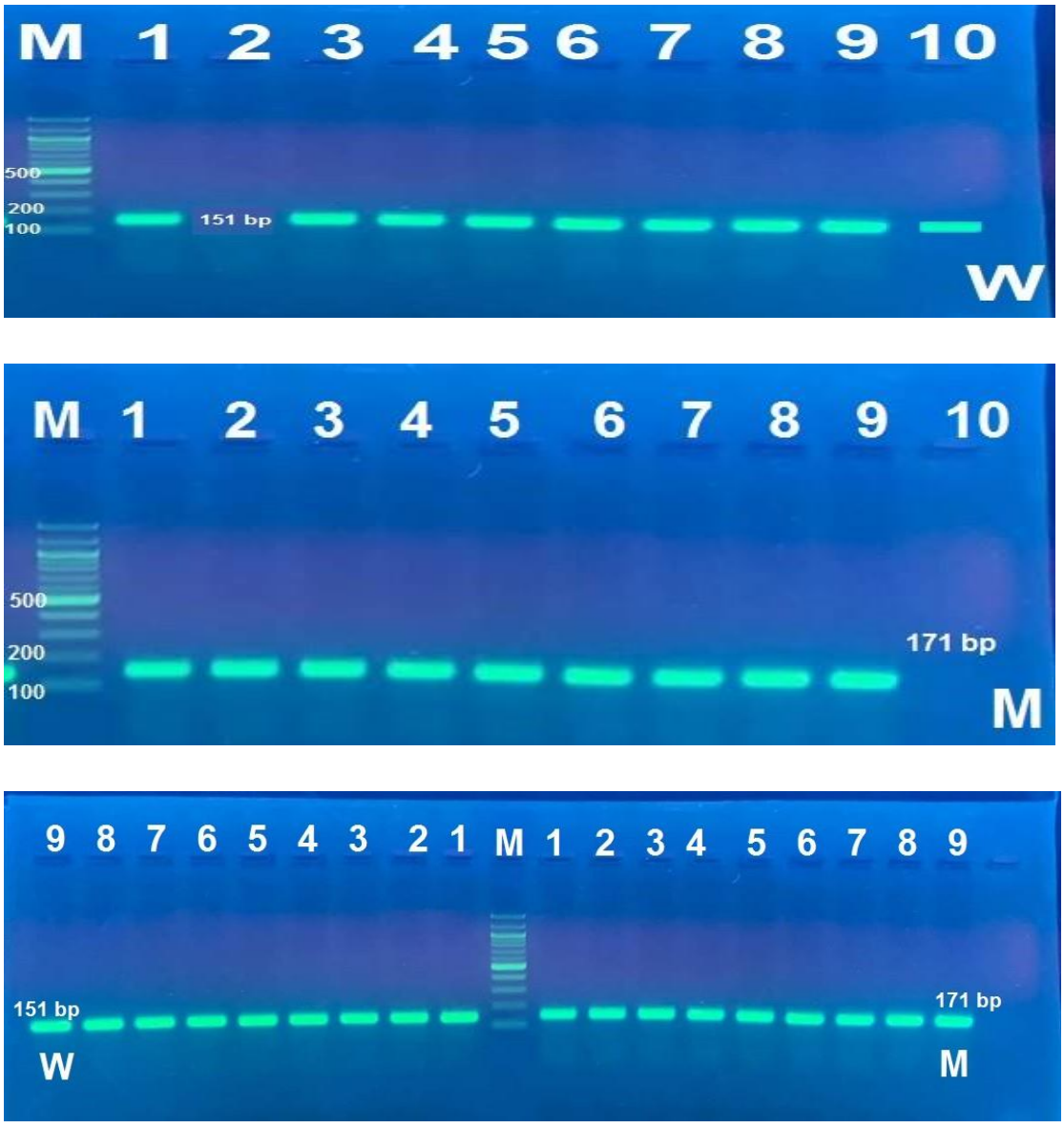
TT homozygous wild , TA -heterozygous, AA -homozygous mutant

P<0.005: OR =(95%CI): [a] Reference

There are significant difference in A,T genotype between patient and control group (p<**0.005**),The A allele is significantly associated with an increased risk of BC, these result were matched to the previous study done by (Solano *et al*., 2012).

Among early onset familial cases, 10-40% was found to be associated with *BRCA1* and *BRCA2* mutations. In contrast, among sporadic early-onset BC patients, the frequency of BRCA1/2 mutation ranges from 1% to 10% (Carraro *et al*., 2013).

Figure (4-6) shows the samples TA (1-9 and 11-19) are heterozygous for this type of mutation, first lanes represent the 1 sample; lanes 10WT (wild type specific amplification, second lanes 151bp fragment and 2MT (mutant-specific amplification 171bp fragment .



Figure(4-6):Represents amplification Products of *BRCA2* ;W: wild-type alleles with 151 bp by wild-type specificprimers (10) with DNA samples in Group M: Mutant-type alleles with 171 bp by mutan -type specificprimers (2) with DNA samples in GroupA. WM,heterozygous(1,3,4,5,6,7,8,9,11,12,13,14,15,16,17,18,19) ,

On 2% agarose gel , red safe stained 5 Ml for 15 min on high (50 volt)&60 min on low. There was a DNA molecular marker in Lane M. (100 bp).

4.4 4. Genotyping of *Foxp3* (rs3761548) polymorphism using PCR-RFLP.

For (*Foxp3*) genotyping, the genomic DNA was amplified using specific primers and accomplished by the Thermo-cycler apparatus under the optimal conditions as mentioned in the table (3-10).The results revealed that the presence a single band (487 bp) of the target sequence of *Foxp3*gene in agarose gel as shown in figure (4-7).

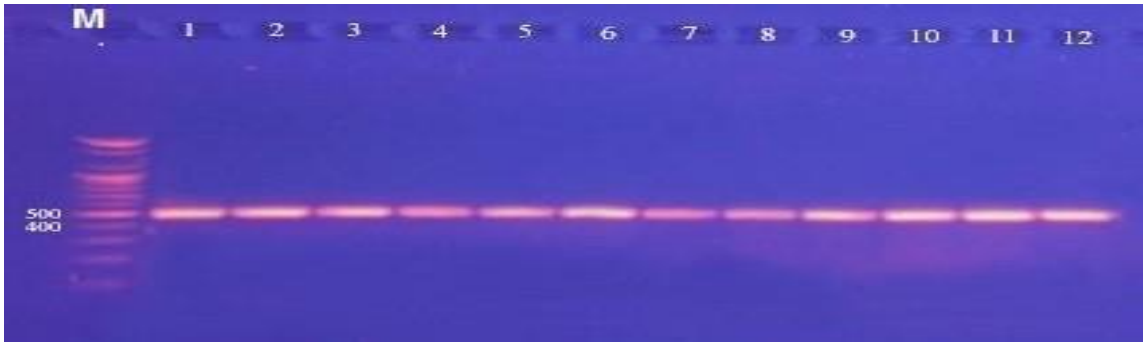


Figure (4-7): Electrophoresis pattern of PCR products of *Foxp3*genes.M: refers to DNA size marker line 1 (100bp) DNA marker, line 1-8 *Foxp3* genotype for patients, line 9-12 *Foxp3* genotype for control. Electrophoresis condition: On a 2% agarose gel, red safe stained 5 Ml for 15 min on high (50 volt)&60 min on low.

The 487-bp PCR product of *Foxp3* A>C was digested with *PstI* restriction endonuclease and the A allele was replaced by the C allele with a loss of digestion site. The homozygous A/A genotype had into 487 bp for the undigested; the heterozygotes mutant A/C genotype was completely digested into 487, 329 and 158 bp and the homozygotes mutant C/C genotype 329 and 158 bp corresponded to the heterozygous A>C genotype Figure (4-8).

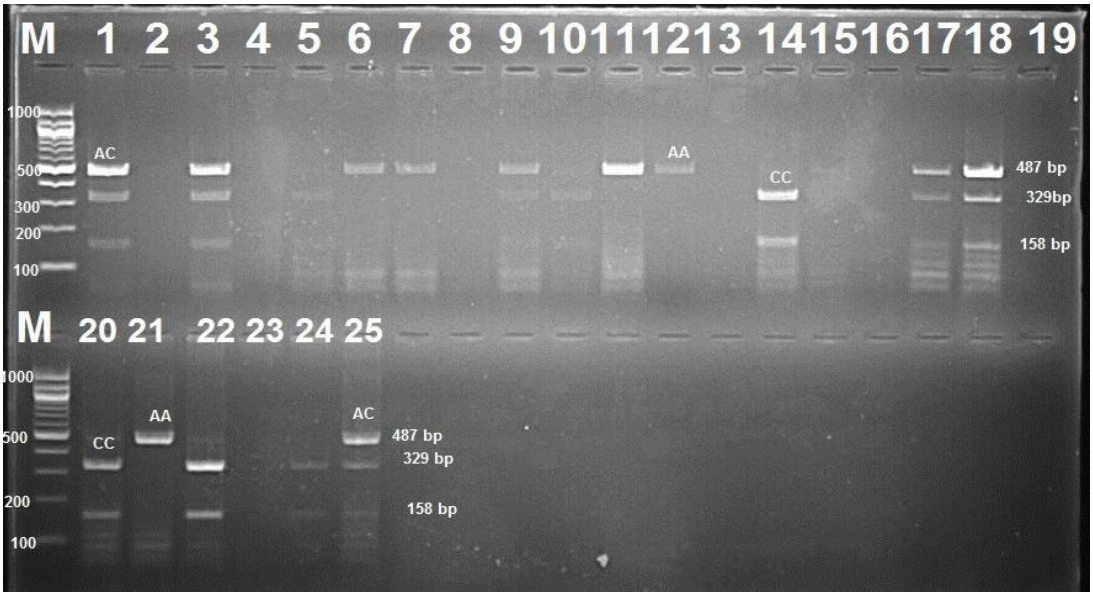


Figure (4-8): Electrophoresis pattern of *Foxp3* genotyping using RFLP technique digested with *PstI* restriction enzyme ,On a 2% agarose gel, red safe stained 5 Ml for 15 min on high (50 volt)&60 min on low.**),** M; refers to DNA size marker(100bp) lane, (5,8,9,11and 21) A/A homozygous genotype; (1,3,18,19and 25) A>C heterozygous genotype ; (5,6,7,8,914,20,22and 24) C/C homozygous genotype PCR product**.**

The *Foxp3* genotype showed critical variety between alleles in patients and control, the AA was showed up in 70% control while it was 22% in patients, CC genotype was more successive in patients (52%) than control (24%). AC genotype was continuous in control (6%) than patients (26%), as shown in table (3-5) and figure (4.8).

In present study, it was firstly reported that the role of genetic polymorphisms of *Foxp3* in Breast cancer in Iraq population, that *Foxp3* A/C polymorphisms were associated with the Breast cancer risk, and the C allele of *Foxp3* promoter polymorphisms had significantly increased the susceptibility to Breast cancer in Iraq population. This finding suggested that the *Foxp3* A/C polymorphisms might be used as a genetic marker for the onset and development of Breast cancer in the Iraqi population.

Table (4.5) Distribution of allele frequency and genotype of *Foxp3* in case-control study.

Genotypes	Patients (N=50)		Control (N=50)	OR(95%CI)	P-value
Foxp3	**AA [a],n(%)**	11(22%)	35 (70%)		
	AC ,n(%)	13(26%)	3 (6%)	0.07 (0.01 – 0.30)	0.001 *
	CC,n(%)	26 (52%)	12 (24%)	0.14 (0.05- 0.38)	0.001*
Allele Frequency					
	A, n (%)	35 (0.35)	73 (0.73)	0.19 (0.10- 0.36)	0.001 *
	C, n (%)	65(0.65)	27 (0.27)		

AA-homozygous wild , AC-heterozygous, CC-homozygous mutant

P<0.005`:OR =(95%CI): [a] Reference

There were data suggested significant associations between rs3761548A>C and various types of cancer including colorectal , thyroid lung and hepatocellular cancers (He *et al.*, 2013, Jahan *et al*., 2014, Jiang *et al.*, 2015,Ramachander *et al*., 2016)

Jiang and Ruan (2014) study in Indian women revealed a highly significant association between AA genotype of rs3761548, with the advanced stage (T3–4) of the tumor. They also showed AA as a risk genotype for fast progression in the premenopausal group (Shen *et al.*, 2010) However, our findings are not in accordance with the studies that revealed a lack of association between rs3761548 polymorphism and BC (Pan *et al*., 2020)

As the promoter region is the site for transcription factor binding, polymorphisms in this region of the *FoxP3* gene may potentially change the binding specificity of transcription factors and modify the kinetics of transcription initiation, which result in *FoxP3* gene expression alteration (Zheng *et al.*, 2013) . There is a good agreement between the above theory

and the earlier reports about the significant number of *FoxP3* genes mutations , dysregulated *FoxP3* expression in BC cells and effects of promoter region polymorphisms on *FoxP3* expression levels. (Al- Hajaj and AL-Battat ,2015)

Conclusions and Recommendations

1.Conclusions:-

1- The results show that significant effect between age and breast cancer.

2- No significant effect for marital state.

3- Found significant effect for menopause .

4- The higher incidence of breast cancer in in Family history is related to many factors which have collectively a cumulative effects including reproductive factors, genetic factors, hormonal factors, exposure to radiation and life style.

5- Family history of breast cancer indicates a strong association with risk of developing breast cancer..

6- In *BRCA1* gene Polymorphism An increased frequency of homozygotic mutant genotypes (GG) of patients compared to controls.

7- In *BRCA2* The A allele is significantly associated with an increased risk of BC.

8- *Foxp3* polymorphisms had significantly increased the susceptibility of Breast cancer .

9- significant effects were found IL12 levels and breast cancer patients.

2.Recommendations:-

1. Adopting early diagnosis of breast cancer, which is one of the most important methods of prevention and reduce the resulting mortality rates.
2. Conducting periodic examinations for women in order to record any abnormal signs in the breast because of its importance in the early diagnosis of the disease.
3. Adopting the molecular examination periodically in the examination of genes for reviews to the center Early detection in Iraqi hospitals in order to early detect a defect in the the main genes causing breast cancer.
4. Study other genetic polymorphisms (site) of *HLA-G*, *TGFβ3*, and *TGFβRIII* genes in Iraqi patients with breast cancer.
5. Investigation of other biomarkers for diagnosis and monitoring of Breast cancer with complication.
6. Use more than one diagnostic marker for diagnosis of Breast cancer.
7. Use FOXP3 as indicator for Reduced immunity and the incidence of breast cancer

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خلاصة

سرطان الثدي هو السرطان الأكثر شيوعاً الذي يصيب النساء في جميع أنحاء العالم. وقد تناولت دراسات مختلفة العوامل المسببة لهذا السرطان بهدف إيجاد طريقة للتشخيص المبكر والعلاج للمرضى .

أجري هذا البحث على ٥٠ مريضة (جميعهن إناث) تم التأكد من إصابتهن بسرطان الثدي من خلال الفحوصات النسيجية المرضية لمريضات مستشفى مرجان في مدينة الحلة.

أجريت الدراسة في الفترة ما بين أيلول ٢٠٢٢ إلى تموز ٢٠٢٣ في كلية العلوم للبنات /جامعة بابل . تم تقسيمهم وفقا للحالة السريرية إلى ٥٠ مريضة و ٥٠ من النساء الأصحاء ظاهريا تم اعتبارهم مجموعة سيطرة.

جميع النساء (المرضى ومجموعة السيطرة) أعمارهم بين ٣٠-٧١ سنة، تم أخذ التاريخ العائلي ، انقطاع الطمث، الحالة الاجتماعية بعين الاعتبار كعوامل خطر .النتائج اظهرت انه لا يوجد تأثير للحالة الزوجية بينما يوجد تأثير لانقطاع الطمث والتاريخ العائلي .

بالإضافة إلى ذلك، تم العثور على علاقة بين تعدد أشكال الحمض النووي لبعض الجينات المختارة وسرطان الثدي والتحقيق في الطفرة الجينية التي قد تكون مرتبطة بسرطان الثدي باستخدام تقنيات نظام الطفرة المقاومة للتضخيم (ARMS -PCR) ونظام (RFLP-PCR) .

لوحظ ان توزيع الانترلوكين١٢ لمجموعه من المرضى ومجموعة السيطرة بواسطة استخدام تقنية فحص الامتصاص المناعي المرتبط بالانزيم ELISA

وكان واضحا انخفاض مستوى انترلوكين ١٢ لدى مرضى السرطان بشكل ملحوظ ($P\leq0.005$)

بحثت الدراسة الحالية دراسة جزيئية لبعض الجينات ذات العلاقة بسرطان الثدي مثل جين (BRCA1,BRCA2,FOXP3)

تظهر نتيجة توزيع الطفرة 185 del AG من جين BRCA1 في مجموعة السيطرة ومرضى سرطان الثدي وكانت كالتالي (52% AA , 16% GG , 32% AG) في مجموعة السيطرة. وكانت (36% ,GG 18% AA , 46% AG) في المرضى .

تم العثور على زيادة تواتر الانماط الجينية الطافرة متماثلة الزيجوت GG في المرضى مقارنة بمجموعة السيطرة . وكان هناك فرق احصائيا في توزيع تكرارات الاليل في المرضى ومجموعة السيطرة .

(AG ٠.٠٠٠٥، OR ٠.٣٢ ، ٩٥% CI ٠.١٨ to ٠.٥٨) في حين وجدت هذه الدراسة ان توزيع جين BRCA2 في مجموعة السيطرة ومرضى سرطان الثدي بان هنالك فرق كبير في التركيب الوراثي A,T بين المرضى ومجموعة السيطرة P≤0.005 ويرتبط الاليل T بشكل كبير مع زيادة خطر الاصابة بمرض سرطان الثدي.

وراثيا تم استخدام تقنية RFLP في جين FOXP3(rs3761548) وهو جين متعدد الاشكال وله تاثير كبير على مرضى سرطان الثدي .وان النمط الجيني لجين FOXP3 له تنوع حرج بين الاليلات في المرضى ومجموعة السيطرة .

وقد ظهر AAفي ٧٠% من الكونترول بينما كان ٢٢% في المرضى ,وكان النمط الجيني CC اكثر تواجدا في المرضى ٥٢% من مجموعة السيطرة ٢٤%,وكان النمط الجيني AC في مجموعة السيطرة ٦% وفي المرضى ٢٦%.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل
كلية العلوم للبنات

دراسة جزيئية لبعض الجينات المرتبطة بسرطان الثدي و تقدير الانترلوكين 12 لدى مرضى محافظة بابل

تم تقديمها الى
مجلس كلية العلوم للبنات – جامعة بابل
كجزء من متطلبات الحصول على درجة الماجستير في علوم الحياة

من قبل

روز عادل حميد السعدي

اشراف

أ. م. د. اشراق عبد الامير صالح المعموري

ربيع الاول /١٤٤٥ هـ

تشرين الاول /٢٠٢٣ م