University of Kerbala
College of Applied Medical Sciences
Department of Clinical Laboratories

Association of *TLR7* Gene Variants with Some Biochemical Markers among Breast Cancer Women

A Thesis Submitted to the Council of the College of Applied Medical Sciences – University of Kerbala In Partial Fulfillment of the Requirements for the Degree of Master in Clinical Laboratories

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

وَلَقَدْ ءَاتَيْنَا دَاوُۥدَ وَسُلَيْمَٰنَ عِلْمًا ۖ
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كَثِيرٍ مِّنْ عِبَادِهِ ٱلْمُؤْمِنِينَ ﴿١٥﴾

صَدَقَ اللَّهُ الْعَلِيُّ الْعَظِيمُ

(سورة النمل ، اية 15)

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I certify the thesis entitled (**Association of *TLR7* Gene Variants with Some Biochemical Markers among Breast Cancer Women)** was prepared under my supervision by **Zahraa Majid Chasib** at the department of Clinical Laboratories\ College of Applied Medical Sciences \ University of Kerbala, in partial fulfillment of the requirements for the degree of Master in Clinical Laboratories.

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Committee Certification

We, the examining committee, certify that we have read the thesis entitled **"Association of *TLR7* Gene Variants with Some Biochemical Markers among Breast Cancer Women"** and have examined the student **(Zahraa Majid Chasib)** in its content and that in our opinion it is accepted as a thesis for degree of Master of **Clinical Laboratories.**

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Approval Certification

We certify that the thesis entitled **Association of *TLR7* Gene Variants with Some Biochemical Markers among Breast Cancer Women** fulfills partial requirements of the degree of Master in Clinical Laboratories.

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Dedication

To *the* ***Prophet Muhammad*** *(may Allah bless him and his family and grant them peace).*

To Imam Mahdi *(may Allah Almighty hasten his relief).*

To My Mother *. She was never an ordinary woman. In my mind, she was and still was embodied as an angel... as a superhero who faces all difficulties ... as a doctor ... as a lawyer... like all the women of the universe at the same time, but; I lost her twelve years ago . To those who I missed the warmth of her applause in joy at my achievement at this moment: from (Zahraa) to (****Amira****) ... to my mother .*

To *the one who taught me to climb the ladder of life with wisdom and patience ...* ***My father*** *.*

To *my support and honorary source: My brother,* ***The engineer (Mortaza)*** *.*

To *my dear* ***friends****, They were sisters that my mother did not give birth to*

(Batool, Nada, Zainab, Rana and Wafaa) .

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Symbol	Description
AP-1	Activating Protein-1
APCs	Antigen-Presenting Cells
BC	Breast Cancer
BCS	Breast Conservation Surgery
BLAST	Basic Local Alignment Search Tool
BMI	Body Mass Index
CA 15-3	Cancer Antigen 15-3
CA125	Cancer Antigen 125
CEA	Carcinoembryonic Antigen
CT scan	Computed Tomography Scan
DAMPs	Damage-Associated Molecular Patterns
DCIS	Ductal Carcinoma In Situ
DNA	Deoxyribonucleic Acid
ER	Estrogen Receptor
EV71	Enterovirus 7I (EV7I) Infection
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
HCV	Hepatitis C Virus
Her-2	Human Epidermal Growth Factor Receptor 2
HFMD	Hand, Foot, and Mouth Disease
HIV	Human Immunodeficiency Virus
IDC	Invasive Ductal Carcinoma
IDC-NOS	Invasive Ductal Carcinomas Not Otherwise Specified
IDC-NST	Invasive Ductal Carcinomas Not Special Type
IHC	Immuno Histo Chemistry
IL	Interleukin
ILC	Invasive Lobular Carcinoma
IRAK	Interleukin-1 Receptor-Associated Kinase
IRF3	Interferon Regulatory Factor 3
LABC	Locally Advanced Breast Cancer
LH	Luteinizing Hormone
MAPK	Mitogen-Activated Protein Kinase
MBC	Metastatic Breast Cancer
MDSCs	Myeloid-Derived Suppressor Cells
MEGAX	Molecular Evolutionary Genetics Analysis X
MRI	Magnetic Resonance Imaging
MUC 1	Mucin 1
NCBI	National Center for Biotechnology Information

NF-κB	Nuclear factor kappa-B cells
nsSNPs	Nonsynonymous variants
PAMP	Pathogen Associated Molecular Patterns
pDCs	Plasmacytoid Dendritic Cells
PET	Positron Emission Tomography
PR	Progesterone Receptor
PRRs	Pattern Recognition Receptors
RNA	Ribonucleic Acid
SARS-CoV-2	Severe Acute Respiratory Syndrome-Corona Virus-2
SLE	Systemic Lupus Eerythematous
SLN	Sentinel Lymph Node
SLNB	Sentinel Lymph Node Biopsy
SNPs	Single Nucleotide variants
ss-RNA	Single Stranded-Ribonucleic acid
TAK1	Transforming growth factor-β Activated Kinase 1
TLR7	Toll Like Receptor 7
TNBC	Triple-Negative Breast Cancer
TNF	Tumor Necrosis Factor
TNFα	Tumor Necrosis Factor Alpha
TRAF6	TNF Receptor Associated Factor 6
UBC	Urinary Bladder Cancer
XCI	X-Chromosome Inactivation

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Summary

The present study as a Case-Control Study was carried out in the Department of Clinical Laboratories / College of Applied Medical Sciences / University of Kerbala during the period from November 2022 to May 2024. The study aimed to shed light on the association between the variant of the *TLR7* gene and serum level of some biochemical markers (CA15-3, CEA, CA125, and CA27-29) in breast cancer patients from Iraqi women. 100 women volunteers enlisted, 50 women with the breast cancer , in contrast 50 women included apparently healthy were enrolled in this study, with an age range of (29-75) years at the time of the investigation.

The blood samples were drawn from volunteers were recruited from AL-Imam AL-Hussein Center for Oncology and Hematology in Karbala city / Iraq , the ethical consent was signed by each volunteer. The study was a population genetic study, carried out on two group samples. The variants of the *TLR7* gene were investigated by the Sanger sequencing method. The serum levels of (CA15-3, CEA, CA125, and CA27-29) were measured by enzyme linked immunosorbent assay (ELISA) technique. The statistical analysis displayed statistically significant differences when the serum levels of the four tumor markers namely (CEA, CA125, CA15-3, and CA27-29) were compared between control and patient groups ($p<0.001$).

The genetic analysis of the results of the 20 samples (selected from patient samples) showed different types of variants in the *TLR7* gene. The number of variants present in the samples was different from one sample to another, the all 20 samples displayed multiple variants in their exon4 region sequences , while 6 out of 20 samples demonstrate double variants and the rest 14 samples displayed multiple variants in intron2 region sequences.

The detected variants were divided into two groups, previously registered variants (4 variants, 1 exon4 and 3 intron2), and novel non-registered variants (13 variants, 8 exon4 and 5 intron2).

The previously registered variants [12888260 G/A] was detected in Exon4 region in 10 out of 20 samples , while variants [12871738 A\G, 12871850 A\C, and 12871888 T\A] were detected in Intron2 region in 18 out of 20 samples. The novel non-registered variants [12888227 T\G, 12888234 T\G, 12888246 T\G, 12888294 A\G, 12888276 A\T, 12888269 G\A, 12888351 A\G, and 12888221 A\C] were detected in Exon4 region in 12 out of 20 samples, while variants [12871741 A\T, 12871749 G\C, 12871759 T\G, 12871764 A\G, and 12871768 G\C] were detected in Intron2 region in 17 out of 20 samples.

The effects of *TLR7* gene variants on study parameters (CA15-3, CEA, CA125, and CA27-29) were investigated and the results showed that the variant [12871749 G/C] of the intron2 region was moderately negatively and statistically significantly correlated with the CEA tumor marker serum levels (point biserial correlation coefficient = -0.396 , p value = 0.03). Furthermore, the statistical analysis showed a statistically significant correlation (p value = 0.015) between the variant [12888221 A/C] of the exon4 region and the CA125 tumor marker serum value, the interaction was of an intermediate correlation (point biserial correlation coefficient = -0.442).

The variant [12871764 A/G] of the intron2 region was moderately associated with the CA15-3 tumor marker levels (point biserial correlation coefficient = -0.385) and the two variables were statistically significantly associated (p value = 0.036).

The results of Exon4 region displayed significant differences between the same groups in the level of CEA (p value = 0.015), while the result displayed non-significant differences between the two groups divided according to number of variants (<6 and >6 variants) in the level of CA125, CA15-3, and CA27-29 with a p value 0.44, 0.96, and 0.48 respectively.

The statistical analysis indicated non-significant differences between new and mixed variants in the level of CEA, CA125, and CA27-29 with a p value 0.76, 0.32, and 0.428 respectively. While the result of CA15-3 displayed significant differences between new and mixed variants where (p value = 0.02) in Exon4 region.

CHAPTER ONE

Introduction

1.1. Introduction

Toll-like receptors gene (*TLRs*) play an important role in immune-surveillance which that play a key role in innate immune activation, cytokine generation, adaptive immune system indirect stimulation. *TLRs* have ten members in the human. *TLR1, TLR2, TLR4, TLR5, TLR6,* and *TLR10* are located on the cell membrane, while *TLR3, TLR7, TLR8,* and *TLR9* are located in endosomes (intracellular vesicles) (Kemball *et al*., 2010; Birra *et al*., 2020).

TLR7 is expressed on A dendritic cell (DC) and monocyte-macrophages, and when activated, they produces IL-6, IL-1, type 1 interferon, and TNF-alpha. SNPs, which can affect a protein's normal function and denature it is structure by affecting its stability, folding form, and ligand-binding site, have been linked to a variety of diseases in previous researches (Khan *et al*., 2017; Dyavar *et al*., 2021).

The most common type of cancer in women and the primary cause of cancer-related mortality is breast cancer. Early detection is a key tactic to enhance outcomes because there are few indications and symptoms in the early stages. It has been demonstrated that the genetic and transcriptome characteristics of breast tumors correlate with their grade (Winters *et al*., 2017).

Long-term reductions in breast cancer death rates could be achieved with early-stage cancer identification. Finding cancer cells in their early stages is crucial for the best prognosis. The pattern recognition receptors that have been investigated the most are toll-like receptors (*TLRs*). Growing data points to a significant correlation between *TLRs* and the incidence and progression of breast cancer (Wang, 2017; Shi *et al*., 2020). The *TLRs* signaling pathway has been demonstrated to be expressed in

tumor cells and immune cells, and it is essential in triggering immunological responses. It has also been linked to the development, of breast cancer and other types of cancer (Toroghian *et al*., 2022) .

TLRs play a pivotal role in initiating both innate and adaptive immune responses by identifying a range of pathogen-associated molecular sequences. Recent data, however, indicates that *TLRs* are also expressed on a broad range of malignancies, indicating potential functions for *TLRs* in tumor biology. Over the past several years, different studies have indicated that *TLR* variants modify the cellular immune response and that some of these variants are associated with susceptibility to infectious and inflammatory diseases as well as cancer incidence and severity (Lu *et al*., 2015). However, previous studies have reported that the variants on the *TLR1-TLR10* cluster have been associated with increased risk of cancer (Pandeyn *et al*., 2019).

On the one hand, *TLR* ligands can suppress tumor growth. On the other hand, *TLR* agonists can promote the survival of malignant cells and increase their resistance to chemotherapy. Furthermore, the ligation of *TLRs* in tumor cells increases the production of immunosuppressive cytokines, such as interleukin (IL)-10, suggesting that tumor cells also utilize *TLR* activation to escape from tumor immune surveillance. Thus, further investigation is mandatory to decipher the role and the genetic variants of *TLRs* in cancer (Shcheblyakov *et al*., 2010).

By controlling metalloproteinase and integrin's, activation of tumor cell *TLR*s to enhances tumor cell invasion and metastasis, In addition to that promotes tumor cell proliferation and resistance to apoptosis. Additionally, proinflammatory and immunosuppressive molecules are synthesized when *TLR* signaling is activated in tumor cells. which enhance the resistance of tumor cells to cytotoxic lymphocyte attack and lead to

immune evasion. Therefore, the neoplastic process may take *TLR* signaling pathways to advance cancer progression, which suggests that targeting tumor *TLR* signaling pathways may open up new therapy options (Huang *et al*., 2008).

1.2. The aim of the study:

2. Investigate some biochemical markers (CEA, CA125, CA15-3, and CA27-29) in breast cancer women.
3. Investigate the *TLR7* variants in breast cancer women.
4. Study the association between of *TLR7* variants with some biochemical markers (CEA, CA125, CA15-3, and CA27-29) in breast cancer women.

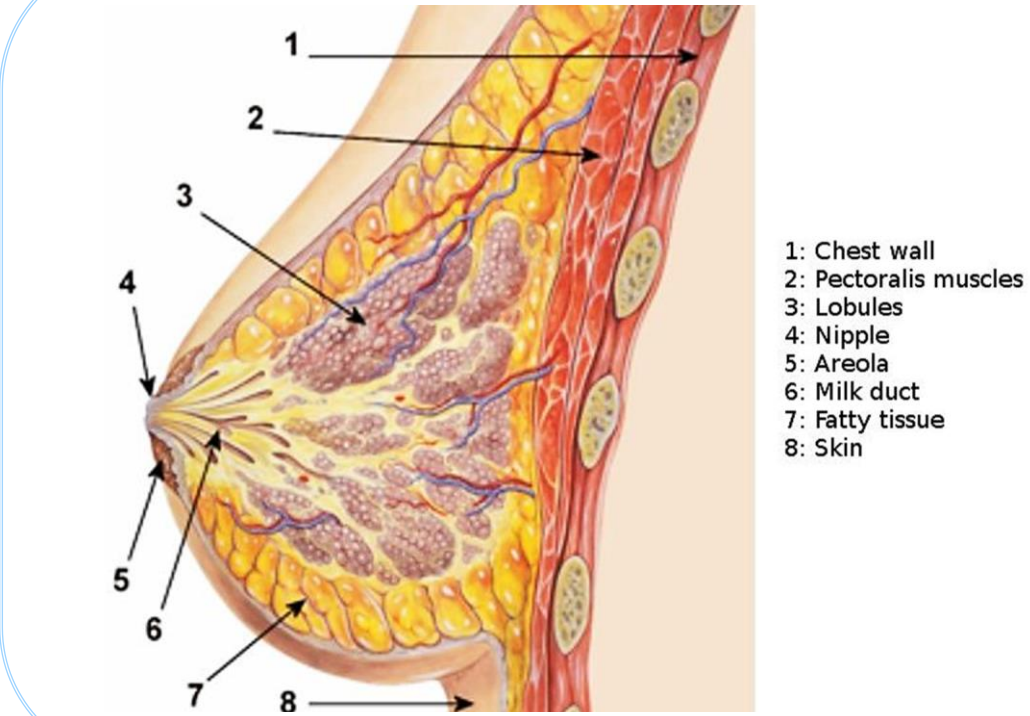
CHAPTER TWO

Literature Review

2. Literature Review

2.1. Anatomy of the Breast

The mammary gland is situated in the pectoral region in the superficial fascia. However, a segment called the "axillary tail of Spence" pierces the deep fascia and lies in the axilla up to the 3rd rib level. The mammary gland extends vertically from the 2nd to the 6th rib. Horizontally, it spreads from the lateral sternal border to the mid-axillary line. Deep to the mammary gland tissue is the retromammary space, a loose connective-tissue plane that gives free mobility to the gland. Below the retromammary space is the pectoral fascia, which covers the pectoralis muscle. The serratus anterior and external oblique are other muscles that lie deep in the mammary gland. The mammary gland is divided into 3 parts: skin, parenchyma, and stroma (Khan et al., 2023).



(Figure 2-1) : Anatomy of the Breast Region (Khan et al., 2023)

2.1.1. Development of the Breast From Birth Until Puberty

During this period the breast consists of lactiferous ducts, with no alveoli. As puberty begins, the circulating estrogen causes the ductal epithelium and surrounding stroma to grow. These ducts begin to arborize and form collecting ducts and terminal duct lobular units. These ultimately form buds that precede further breast lobules. Surrounding the ducts, vascularity increases, and connective tissue increases in volume and elasticity, replacing adipose tissue and providing support for the development of ducts. Between the ages of 8 and 13, beginning breast budding is one of the first signs of adolescence in girls (Bistoni and Farhadi, 2015).

2.1.2. Blood Supply

2.1.2.1. Arterial

The internal thoracic artery's perforating branches from the 2nd to the 6th ICS provide circulation to the medial gland regions. The lateral thoracic artery supplies the superolateral breast parenchyma. The axillary artery's superior thoracic, thoracoacromial, subscapular, and thoracodorsal branches supply a portion of the superior breast parenchyma. The musculophrenic artery originates from the internal thoracic artery and supplies inferior breast segments. The branches of the anterior and posterior intercostal arteries penetrate the chest wall muscles and supply the deep central breast parenchymal tissues (Khan et al., 2023).

2.1.2.2. Venous

Breast veins are divided into superficial and deep veins. Superficial veins commonly drain the central and peripheral breast areas. The central veins form a venous plexus known as the "circulus venosus of Haller". Blood flows from this venous network into the internal thoracic vein

medially, lateral thoracic veins laterally, and the superficial neck veins superiorly. The deep breast veins drain into the internal thoracic, axillary, and posterior intercostal veins (Khan et al., 2023).

2.1.3. Nervous

the lateral cutaneous branches of the third through sixth intercostal nerves provide the majority of the breast sensory supply of nerves. Certain cutaneous branches of the cervical plexus supply some of the sensory innervation in the cranium. The fourth intercostal nerve innervates the nipple-areola complex (Skandalakis, 2009).

2.1.4. Axillary Lymph Nodes

Within the axilla are multiple sets of lymph nodes. These nodes can be divided into two groups: the lymph nodes of the axillary vein, which run along the axillary vein between the humerus and the pectoralis minor, and the apical or subclavicular nodes, which are situated medial to the pectoralis minor muscle. The interpectoral or Rotter's nodes lie between the pectoralis major and minor muscles. The central axillary nodes are found beneath the border of the pectoralis major muscle and below the pectoralis minor. The external mammary nodes lie over the axillary tail of the Spence. Intramammary lymph nodes and paramammary lymph nodes are located in the fat layer over the upper, outer quadrant of the breast. For surgical dissection purposes, there are three lymph node levels of the axilla, which are all defined by their relationship to the pectoralis minor muscle :-

Level I: The nodes are located laterally to the edge of the pectoralis minor. This level includes lateral axillary, subscapular, and external mammary lymph nodes.

Level II: The nodes are situated behind the pectoral minor. This level

includes the central axillary lymph nodes.

Level III: Nodes are medial and superior to the pectoralis minor. This level includes the sub-clavicular or apical lymph nodes (Harris *et al*., 2012).

2.2. Physiological Breast Development

The mammary gland shows physiological variants in size, shape, contour, density, and spacing. These variants depend on the factors as age, height, weight, genetic composition, race, nourishment, and environment. The human breast consists of parenchymal and stromal elements. The parenchyma forms a system of branching ducts eventually leading to secretory acini development and the stroma consists mainly of adipose tissue, providing the environment for the development of the parenchyma. Breast development occurs in distinct stages via complex epithelial-mesenchymal interactions, orchestrated by signaling pathways under the regulation of systemic hormones. Many hormones that are unregulated in the early stages of puberty contribute to breast development. Progesterone and estrogen are the main hormones responsible for breast growth and development during this time. Whereas estrogen stimulates the development of ducts, progesterone stimulates the production of lobules and epithelial differentiation (Javed and Lteif, 2013; Khan et al., 2023).

2.3.1. Breast Cancer

Globally, The second most prevalent type of cancer in women is breast cancer (BC). Many studies have demonstrated the impact of environmental and lifestyle factors such as (alcohol consumption, high-fat diets, and sedentary lifestyles) on the development of mammary gland cancer. Lowering the morbidity and death rate of the disease may be possible by reducing these causes (primary prevention). Secondary prevention, comprising diagnostic tests (e.g., mammography,

ultrasonography, magnetic resonance imaging, breast self-examination, as well as modern and more precise imaging methods), helps in the early detection of tumors or lesions predisposing to tumors (Kolak et al., 2017).

According to estimates, there are 2.3 million new cases of female breast cancer in 2020, accounting for 11.7% of all cancer cases worldwide, surpassing lung cancer as the primary cause of occurrence (Sung *et al.*, 2021).

Family history of the disease is one of the most significant risk factors for breast cancer, indicating that genetic factors are important determinants of breast cancer risk (Antoniou & Easton, 2006). Epidemiological studies estimate that breast cancer is approximately twice as common among first-degree relatives of breast cancer patients, suggesting strongly that genetic factors are important determinants of disease risk (Feng et al., 2018). Breast cancer continues to be the most frequent cancer in women diagnosed globally. The main causes of breast cancer mortality are delayed presentation of the disease, late stage at diagnosis, limited therapeutic options, metastasis, and relapse. The development and progression of breast cancer is a complex and multi-step process that incorporates an accumulation of several genetic and epigenetic alterations. External environmental factors and internal cellular microenvironmental cues influence the occurrence of these alterations that drives tumorigenesis (Thakur *et al.*, 2022).

Breast tissue is composed of lobules that are glands involved in milk production, ducts that connect the lobules to the nipple and connective tissue, fatty tissue, and lymphatic tissue. Breast cancer occurs when there is an unregulated growth of cells within any of the components of the breast, although this occurs most commonly in the lobules. Males can also develop breast cancer, albeit much less frequently.

Breast cancer develops over time and may go through an in situ phase. Whether in situ or invasive, it may be detected during routine self-breast exams, mammographic screening, or once signs or symptoms have developed. Initially, there are no signs or symptoms associated with breast cancer until a palpable, or visible lump develops within the breast. The most common physical sign that can be appreciated is a painless breast lump. Swollen and enlarged lymph nodes may be present within the axillary region during the early stages of metastasis. Evidence of bloody nipple discharge, heaviness, redness, swelling, breast deformity, or retractions are less common signs, but a substantial indicator of breast malignancy and maybe more evident with advanced stages of disease. As, there are few early signs, it is recommended that all females follow the current breast cancer screening guidelines to identify those with abnormal breast tissue during the early evolutionary stages (Winters *et al*., 2017).

Most breast cancers begin in the ducts or the lobules (Lobules are the milk producing glands, and ducts carry the milk to the nipples) and based on the metastatic spread, they can either be benign or invasive. Ductal carcinoma in situ (DCIS) is considered as non-invasive and early-stage breast cancer confined to the milk ducts. If cancer originates in the ducts or lobules and metastasizes, they are considered invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) respectively. Almost, 80% of breast cancers belong to the IDC category (Henry and Cannon, 2019).

2.3.2. Breast Cancer Staging and Classification

According to existing research, the heterogeneous nature, breast cancer can be further classified into molecular subtypes according to a range of molecular, genetic, and clinical features. Additionally, breast cancer has been categorized into molecular subtypes based on

immunohistochemical factors which include progesterone receptor (PR), estrogen receptor (ER), Ki67 proliferation index, and human epidermal growth factor receptor 2 (Her-2) (Turner et al., 2021; Burstein *et al*., 2021).

Breast cancer staging is determined by tumor size, nodal involvement, the presence of metastases, and specific biomarkers. After a histologic diagnosis of breast cancer, all pathology samples should be tested for estrogen receptors, progesterone receptors, and ERBB2 (formerly HER2) status. Breast cancers that express none of these markers are referred to as triple-negative breast cancers (TNBC) (Trayes and Cokenakes, 2021).

Triple-negative breast cancer (TNBC) is a subtype of breast cancer defined by negative expression of the progesterone receptor (PR) and estrogen receptor (ER), and lack of amplification of the human epidermal growth factor-2 (HER2) gene. It is important to note that TNBCs often exhibit more aggressive behavior compared to other subtypes of breast cancer. (Li *et al*., 2017).

Ductal carcinoma in situ (DCIS) is stage 0, noninvasive breast cancer. Early invasive cancer describes stages I, IIa, and IIb, and locally advanced describes stages IIIa, IIIb, and IIIc. All of these stages of breast cancer are nonmetastatic. Stage IV is metastatic breast cancer (Amin *et al.*, 2017).

2.3.2.1. Stage Zero (Noninvasive Breast Cancer)

a. Lobular Carcinoma in Situ is an incidental microscopic finding of abnormal tissue growth in the breast lobules. It does not progress to but increases the risk of, subsequent invasive breast cancer in either breast by approximately 7% over 10 years. Conversely, ductal carcinoma in situ (DCIS) can progress to invasive breast cancer (Maughan *et al*., 2010). In the past, lobular carcinoma in situ is considered a malignancy. However,

despite its name, it is a proliferative disease that increase the chance of risk breast cancer in the future rather than a carcinoma (Trayes and Cokenakes, 2021).

b. Ductal Carcinoma In Situ (DCIS) is a form of pure, noninvasive carcinoma and is most commonly identified by mammography showing microcalcifications confined to the breast ducts. If left untreated, up to 40% of DCIS cases will progress to invasive breast cancer. Treatment options for DCIS include radiation, lumpectomy (with a target surgical margin of 2 mm), and mastectomy (Cowell *et al*., 2013; Morrow *et al*., 2016).

At the time of mastectomy, a sentinel lymph node (SLN) biopsy is performed to identify the (unlikely) possibility of lymph node involvement. Radiation therapy is offered to patients having a lumpectomy; this combination of lumpectomy with radiation is considered a breast-conserving therapy. Radiation therapy may not be necessary for patients whose low-grade lesions are assessed to have a low risk of recurrence. Radiation therapy is not advised for patients who are treated with mastectomy (Qiao *et al*., 2021).

2.3.2.2. Stages I and II: Early-Stage Invasive

Modified radical mastectomy has typically been the standard of care for early-stage invasive breast cancers. However, breast-conserving surgery has been favored more lately. This therapy includes the tumor being removed without removing excess healthy breast tissue. Following breast-conserving surgery, radiation therapy improves cancer-specific survival rates to levels comparable to mastectomy and reduces local recurrence (Maughan *et al*., 2010).

2.3.2.3. Stage III: Locally Advanced

Tumors greater than 5 cm, regional lymph node involvement, direct involvement of underlying chest wall or skin, tumors considered inoperable but without distant metastases, and inflammatory breast cancer are all considered to be locally advanced breast cancers (LABC). Induction chemotherapy followed by local therapy (radiation therapy, surgery therapy, or both) is becoming the standard of care (Maughan *et al*., 2010).

2.3.2.4. Stage IV, Metastatic Breast Cancer

Metastatic breast cancer is rarely cured, survival is now 24 to 40 months. Minimizing symptoms, extending life, and preserving quality of life are the main objectives of treatment. Targeting the respective appropriate breast cancer subtypes by offering endocrine therapy, chemotherapy, and immunotherapy may be provided. Stage IV breast cancer frequently results in liver, lung, and brain metastases (Peart, 2017; Caswell-Jin et al., 2018).

2.3.2.4.1. Metastasic Pathways

Metastasis is the multiple process by which an original primary tumor develops into a distal secondary tumor. It is a representative hallmark of cancer and leads to treatment failure, leading to the death of many patients (van et al., 2013). Therefore, the patient’s prognosis is closely related to metastasis. The diagnosis of metastatic cancer is considered the final stage in most cancer types. Metastasis is highly complex and involves multiple cellular mechanisms including division from the primary tumor, invasion, evasion of immune surveillance, and regulation of the tissue microenvironment. Some study showed it is possible for cancer cells to spontaneously disseminate to lymph nodes and then escape the lymph node to seed another metastatic site. also, there are other dissemination methods

to distant metastasis (e.g., direct invasion of primary tumor blood vessels) or other methods of escape from the lymph node (e.g., escape through efferent lymphatic vessels) (Park, et al.,2022).

2.3.3. Breast Cancer Diagnosis

2.3.3.1. Screening

Breast cancer is generally diagnosed through either screening or a symptom (e.g., pain or a palpable mass) that prompts a diagnostic exam. Screening of healthy women is associated with the detection of smaller tumors, have lower odds of metastasis, are more amenable to breast-conserving and limited axillary surgery, and are less likely to require chemotherapy. The only screening modality proven to reduce breast cancer-specific mortality is mammography. The potential negative aspects of screening mammography are false positive examinations, radiation exposure, pain, anxiety, and other negative psychological effects (Berry et al., 2005; Pace and Keating, 2014; Fuller et al., 2015).

2.3.3.2. Pathologic Assessment

a. Evaluation and Processing of Specimens

In clinical practice, core biopsy, fine-needle aspiration, or surgical excision are typically used to obtain diseased tissue. Differentiating between closely related illnesses, like ductal cancer and lobular cancer, or atypical ductal hyperplasia, presents a diagnostic challenge for pathologists. The size of the tumor is determined by careful clinical and pathologic correlation. When breast cancer presents as a distinct mass outward from the point of origin, the size is determined by imaging and gross pathologic evaluation. When a tumor forms in a poorly defined field of genetic instability and there is intratumoral normal tissue, determining

its exact size can be challenging (McDonald *et al*., 2016).

b. Predictive Tumor Markers.

Protein expression tests unaffected by tumor morphologic characteristics are used to make critical treatment decisions. Immuno Histo Chemistry (IHC) analysis of paraffin sections is routinely performed for the evaluation of estrogen receptor (ER), progesterone receptor (PR), and Her-2/neu (HER2) status. Although widely used to predict responses to targeted agents, histologic tumor markers are limited by significant intratumoral variation, even within a single biopsy specimen. Routine paraffin-embedded tissue samples can also be examined for RNA and DNA, and in situ hybridization can identify HER2 amplification as a stand-alone assay or as a confirmation test for IHC. In early-stage breast cancer, gene assays are used to predict the risk of distant recurrence and to influence decisions for systemic therapy. Evaluation of ER and proliferation-related genes, like Ki-67, is important to these tests (McDonald *et al*., 2016).

c. Imaging and Staging

For local-regional staging, usually a physical examination, mammography, or ultrasound performed as part of the diagnostic work-up of a patient with newly diagnosed breast cancer is sufficient. It is occasionally advised to get Magnetic Resonance Imaging (MRI), especially if the patient is younger, there may be a genetic variant or multifocal disease present, or the results of an ultrasound or mammography are indeterminate. Magnetic resonance imaging (MRI) can help evaluate the extent of the disease when radiation to the tumor bed is restricted or limited to the region where the nodes are located (Dorn *et al*., 2013).

Furthermore, patients undergoing neoadjuvant chemotherapy may benefit from a breast MRI to assess response and support surgical planning. A chest radiograph and routine laboratory blood tests are sufficient for evaluating staging in patients with clinical stage I or II breast cancer who do not exhibit specific symptoms of metastatic disease. For suspected advanced (stage III or IV) disease, the National Comprehensive Cancer Network guidelines indicate either chest, abdomen, and pelvis CT or chest CT with abdomen and pelvis MRI with bone scan (McDonald *et al*., 2016).

2.3.4. Breast Cancer Treatment

A. Surgery

Surgical intervention remains the means of local and regional breast cancer treatment. As initially reported by William Stewart Halsted in 1894, women with breast cancer are frequently treated with radical mastectomy. Breast conservation surgery (BCS) is pioneered by Fischer *et al*.,(2014), who reported that survival with lumpectomy and radiation is equivalent to that with mastectomy in the treatment of early breast cancer. Improved breast cancer screening resulted in diagnoses of nonpalpable cancers, necessitating the development of a localization approach for surgical treatment. (Fischer *et al*., 2014).

a. Breast Conserving Approaches

Breast conservation surgery (BCS) relies heavily on wire localization of a breast tumor. This procedure is performed by a breast imaging radiologist on the day of surgery. Tumor location and cosmetic considerations influence where the surgical incision should be made on the breast. For a tumor 1-2 cm from the areolar margin, a circumareolar position is optimal, but when a tumor is more than 2 cm from the areola, it

may be beneficial to make an incision directly over the region of concern so that the lumpectomy site can be easily detected (McDonald *et al*., 2016).

b. Non Breast Conserving Approaches

Most women who have early-stage breast cancer that is discovered through screening have the option of having a mastectomy. However, women who have received radiation therapy to the affected side (for prior breast cancer or Hodgkin lymphoma) or who have a relatively small breast may need a mastectomy in the event of large initial breast cancer, extensive calcifications, or multi-centric disease. For in situ or stage I and II invasive cancers, nipple-sparing mastectomy is usually safe oncologically (Peled *et al*., 2016; McDonald *et al*., 2016).

c. Axilla Staging Procedure

The development of sentinel lymph node biopsy (SLNB), which replaced the conventional axillary node dissection, is one of the most significant technological advancements in breast surgery. When the results are negative, the method had an accuracy above 98%, meaning that further dissection is not required. In women with no palpable adenopathy and one or two positive sentinel nodes, whole axillary dissection is not useful for bettering local-regional control or survival when, SLNB results are positive (McDonald et al., 2016).

B. Medical Oncology

There are broad classes of drugs for treating breast cancer are available. The features of the tumor and the severity of the disease determine whether systemic chemotherapy, endocrine therapy, or HER2-directed therapy is recommended for the treatment of breast cancer. For early-stage breast cancer, these features are ER, PR, and HER2 status;

lymph node involvement; and tumor size. For stage IV disease, the receptor status and the locations of metastatic sites are the main factors. (McDonald *et al*., 2016).

C. Chemotherapy.

After definitive surgery, adjuvant chemotherapy is typically advised for patients whose disease has a high risk of recurrence. The pathologic features could be signs that chemotherapy is necessary such as HER2-positive; greater tumor size; positive lymph nodes; ER-, PR-, and HER2-negative. Anthracyclines and taxanes should be a part of cytotoxic therapy for patients with high-risk diseases. It is increasingly frequent to skip anthracyclines for low-risk diseases. Chemotherapy should be chosen after weighing the potential survival benefit against the patient's comorbidities and risk of side effects (McDonald *et al*., 2016).

D. HER2-Directed Therapy

Trastuzumab, a HER2-specific monoclonal antibody, should be administered in addition to chemotherapy to patients with HER2-positive breast cancer as it improves the survival of patients with early-stage breast cancer (Perez *et al*., 2014). Nonanthracycline, taxane-containing regimens can be employed instead of anthracycline and trastuzumab-containing regimens due to the increased risk of heart failure. Trastuzumab should be continued for a year, regardless of the chemotherapy used, and cardiac monitoring should occur every three months (Tolaney et al., 2015; Mavroudis *et al*., 2015).

E. Endocrine Therapy

A patient should get endocrine therapy, such as an aromatase inhibitor, if Patients with ER- or PR-positive breast cancer. Tamoxifen may be recommended if there is a concern over an elevated risk of osteoporosis or aromatase inhibitor intolerance (McDonald *et al*., 2016).

F. Therapy for Metastatic Disease

In the context of metastatic disease, the goal of therapy is to life extension while reducing side effects or symptoms because the metastatic disease is not believed to be curable. Patients with ER- or PR-positive and HER2-negative breast cancer usually get endocrine therapy several times before beginning single-agent chemotherapy. The only available treatment for people with ER-, PR-, and HER2-negative breast cancer is chemotherapy. Patients with metastatic disease should have clinical and imaging examinations, such as CT, bone scans, or PET/CT scans, performed at predetermined intervals to evaluate the response to therapy, regardless of the course of treatment (McDonald *et al*., 2016).

G. Radiation

The low rates of local recurrence in the modern era are due to progress in the multidisciplinary care of breast cancer:

Treatment of disease at an earlier stage because of detection by screening; improved imaging enabling appropriate patient selection for breast conservation; improved surgical techniques and margin pathology assessment; and improved radiation techniques. Radiation also has a proven role in the treatment of stage 0 breast cancer (ductal carcinoma in situ); 90%–95% long-term local control has been achieved with improved patient selection and surgical and radiation techniques.

Modern breast cancer care has advanced to the point where there are low rates of local recurrence. These advancements include better imaging that allows for patient selection that is appropriate for breast preservation, better surgical techniques, evaluation of the margin pathology, enhanced radiation treatments, and early disease treatment as a result of screening-based detection. Additionally, radiation therapy has been shown to be effective in treating stage zero breast cancer, or ductal carcinoma in situ. With better patient selection, surgical, and radiation procedures, There is now 90–95% long-term local control (Gradishar *et al*., 2014).

The past decade has seen considerable advances in the delivery of postoperative radiation that aim to optimize the treatment for each person's anatomy and reduce acute or long-term toxicity. Radiation therapy has a role in the regional control of nodal disease in many patients with high-risk or node-positive stage II, and most patients with stage III, breast cancer (McDonald *et al*., 2016).

In women treated by mastectomy, radiation is recommended for adjuvant treatment when there are clinical or pathologic factors predicting an intermediate to high risk of local–regional recurrence (Gradishar *et al*., 2014).

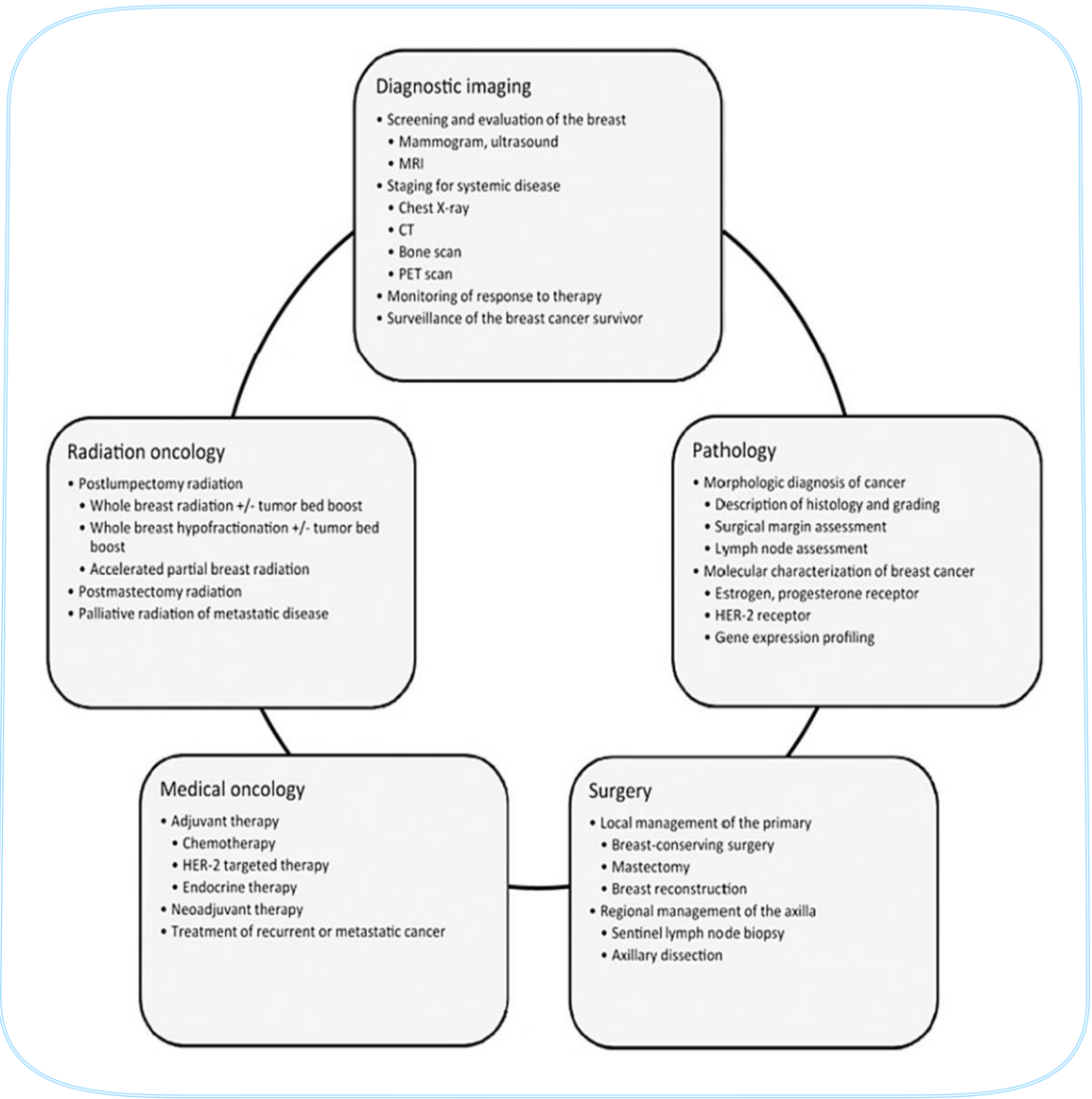


Figure 2-2: Overview of Multidisciplinary Breast Cancer Management. (McDonald *et al.*, 2016).

2.3.5. Biochemical Markers of Breast Cancer

Despite an increase in the incidence of breast cancer, the prognosis for the disease has improved due to early detection, the use of efficient systemic adjuvant therapy, and adherence to prognostic variables (De Cock *et al.*, 2021). Tumor size and tumor grade are two significant prognostic indicators for breast cancer that are frequently utilized (Barzaman *et al.*, 2020).

However, according to Duffy et al. (2017), prognostic biomarkers of tumor size, tumor grade, and lymph node metastases are insufficient for the appropriate care of individuals with breast cancer who are discovered early. Despite an increase in the incidence of breast cancer, the prognosis for the disease has improved due to early detection, the use of efficient systemic adjuvant therapy, and adherence to prognostic variables (De Cock *et al.*, 2021). Serum tumor indicators like carcinoembryonic antigen (CEA), cancer antigen 125 (CA125), and cancer antigen 15-3 (CA15-3) are used in the clinical practice of breast cancer are widely utilized. These have been created as noninvasive, readily accessible, and reasonably priced tumor indicators for breast cancer that can be used for early detection, tracking, and prediction (Ma *et al.*, 2020).

2.3.5.1. Cancer Antigen 15-3 (CA15-3)

Cancer antigen 15-3 is an antigen expressed in benign and malignant breast ductal epithelium. It is a mucin belonging to a large family of glycoproteins encoded by the Mucin1 (MUC1) gene, that are heterogeneously expressed on the apical surface of normal epithelial cell types, including those of the breast (Prabasheela and Arivazhagan, 2011). To monitor patients with metastatic breast cancer while they receive treatment and to detect tumor recurrence in the preclinical stages, serum CA15-3 is utilized as a surrogate marker of disease size. CA15-3 is elevated in a proportion of breast cancer patients with distant metastases. CA15-3 is an additional tool for evaluating the therapeutic response of advanced disease and is used to predict the identification of recurrences in breast cancer patients (Hashim, 2014).

The high levels of CA15-3 would be a solid prognostic marker for Iraqi women with breast carcinoma since preoperative serum concentrations of CA15-3 appear to have a significant relationship to the outcome of patients with early breast carcinoma as it is directly related to advanced stages and recurrence. This connection might also be important when choosing which patients to treat with adjuvant therapies (Hashim, 2014). According to some studies, abnormal CAI5-3 indicates a markedly elevated risk of recurrent breast cancer (Keshaviah *et al*., 2007). Preoperative serum tumor marker testing is therefore valuable, particularly for patients with advanced-stage breast cancer, and may help patients with breast cancer make decisions about their course of treatment (Hashim, 2014).

The most well-researched blood biomarker for breast cancer is CA15-3, a soluble version of transmembrane glycoprotein mucin1, which has different quantities of tandem repeats of peptides modified by glycosylation. Glycosylation alterations are a telltale sign of cancer development. Numerous studies suggest that aberrant glycosylation is a sensitive indicator of carcinogenesis. It is believed that mislocalization of glycosyltransferases and altered expression of chaperone and glycosyltransferase genes are linked to glycosylation alterations associated with cancer. Given that CA15-3 is a highly glycosylated protein, modifications in its glycosylation have the potential to reflect carcinogenesis. Previous research has linked changes in CA15-3 glycosylation to breast cancer (Choi *et al*., 2018).

Given that CA15-3 has a role in cellular adhesion and cell-to-cell interaction, the increase in MUC1 expression on the cell surface may precipitate invasion and metastasis (Araz *et al*., 2019).

2.3.5.2. Carcinoembryonic Antigen (CEA)

In clinical practice, carcinoembryonic antigen (CEA), the most often used tumor marker, is a member of a family of similar cell surface glycoproteins. According to (Shao *et al*., 2015), it is a tumor marker for lung, breast, colorectal, and gastrointestinal cancers. The glycoprotein CEA, which is involved in cell adhesion, is raised in a variety of malignancies. Numerous investigations on primary breast cancer have discovered that higher CEA levels at diagnosis indicate a poor prognosis (Seale and Tkaczuk, 2022). Tumor metastasis is linked to increased blood levels of carcinoembryonic antigen (CEA), a type of cell adhesion molecule. Breast cancer molecular subgroups differ in preoperative CA15-3 and CEA levels, which provide powerful prognostic information for breast cancer patients. Prior to surgery, measuring CEA and CA15-3 levels may be able to predict the prognosis of breast cancer patients and provide them with a customized treatment plan (Li *et al*., 2020).

Breast cancer's persistently rising CEA level could be the cause of the disease's resistance to therapy or its recurrence following it. Additionally, individuals with metastatic or advanced cancer may have greater CEA levels than those with localized illnesses (Shao *et al*., 2015). Elevated CEA is linked to metastatic illness in breast cancer. It has been demonstrated that preoperative CEA readings are stage dependent and correlate with pathological stage and tumor extent. The size of the original and metastatic tumors directly correlates with the circulating levels of CEA in patients with breast cancer. Other, more focused markers, like CA 15-3, are replacing CEA in the detection of breast cancer (Kabel, 2017).

2.3.5.3. Cancer Antigen 125 (CA125)

The MUC16 gene produces the cancer antigen 125 (CA125), which is a major regulator of several cell survival pathways in breast and ovarian cancer cells (Li *et al*., 2020). Carcinoembryonic antigen (CEA) and cancer antigen-125 (CA125) have been commonly employed parameters in clinical follow-up care and monitoring therapy indicated for patients with breast cancer, and circulating tumor markers may be somewhat beneficial (Jafarpour-Sadegh *et al*., 2015).

Patients who had elevated levels of CA125 are at a higher risk of developing lung metastases or surrounding costal bone. Prognosis in patients with stage IV or recurrent breast cancer has been linked to CA125, and increased CA125 in breast cancer patients may be linked to the development of gynecological oncology in the future (Li *et al*., 2019).

2.3.5.4. Cancer Antigen 27-29 (CA27-29)

One protein antigen that contains carbohydrates, CA27-29, is used as a tumor marker for breast cancer. Breast carcinoma-associated antigen is another name for it. The MUC-1 gene is the source of it. Given that 80% of women with breast cancer had elevated levels of CA 27-29, there is a strong correlation between the two conditions. Nonetheless, CA 27-29 has also been detected in patients with ovarian cysts, benign diseases of the kidney, liver, and breast, and patients with various cancers. Elevation of this marker is therefore not organ-specific (Kabel, 2017).

Clinical performance of CA27-29 is comparable to that of CA15-3 in breast cancer patients. Research suggested that CA27-29 might be a less specific but more sensitive marker than CA15-3, but this hasn't been proven conclusively, and most clinicians believe they are basically interchangeable for the majority of clinical uses (Graham *et al*., 2014).

When evaluating breast cancer, CA15-3 and CA27-29 are frequently tested as tumor markers. The MUC1 gene, which produces the mucin glycoprotein, is the source of both the CA15-3 and CA27-29 antigens. The majority of glandular epithelia and the surfaces of ductal cells contain mucin, a glycoprotein. These glycoproteins have a physiological role in lubricating and protecting cells (Lin and Genzen, 2018).

These glycoproteins may be released into the bloodstream as a result of cell polarity disturbance in some cancers, such as kidney, liver, and breast cancers (Nath and Mukherjee, 2014). Consequently, increased serum concentrations of CA15-3 and CA27-29 have been linked to the development of cancer. Additional details about the condition of cancer patients may be found in CA27-29. For example, a persistent increase in the circulating concentration of these markers may suggest an inadequate response to cancer therapy in patients with ovarian or breast cancer (Sturgeon *et al*., 2008).

For instance, in patients with ovarian or breast cancer, a sustained rise in the circulating levels of these markers may indicate an insufficient response to cancer therapy. These markers lack the specificity required for cancer screening, diagnosis, staging, and/or sole use in monitoring recurrence following therapy because they can also be increased in healthy individuals and patients with benign diseases. Nonetheless, CA27-29 may be utilized as an auxiliary evaluation to support choices about the management of metastatic breast cancer (in conjunction with medical history, physical examination, and diagnostic imaging) (Van Poznak *et al*., 2015).

2.4. Toll Like Receptor 7 Gene (*TLR7*)

2.4.1. Human Genome

The human genome, A cell's type dictates how many copies of each chromosome in the cell. A human somatic cell contains 46 chromosomes (Figure 2-3). These chromosomes are divided into 23 pairs, the first 22 pairs are autosomes, while the final pair are sex chromosomes. Autosomal chromosomes are referred to as diploids. sex chromosomes, on the other hand, are referred to as Haploid. Haploid cells are those found in germs, such as eggs and sperm. The X and Y sex chromosomes can be paired to form the XX and XY pairs, respectively, in females and males (Pathak and Bordoni, 2024).

DNA sequences classified as coding or non-coding make up the human genome. The sequences that are capable of being translated into mRNA and proteins during an individual's life cycle are known as DNA coding; these sequences make up a small percentage of the genome. The complete genome's DNA sequences that are not present in exons that code for proteins or that are never represented in the amino acid sequences of proteins that are produced are referred to as DNA non-coding. The majority of the genome is occupied by these sequences (Park et al., 2014).

Every chromosome includes a constriction point known as the centromere and splits the chromosome into two halves, or "arms." The chromosome's short arm is called the (p arm.). The chromosome's long arm is called the (q arm.) (Figure 2-4). Each chromosome's centromere determines the chromosome's distinctive shape and can be used to pinpoint the location of particular genes (Alberts et al., 2015).

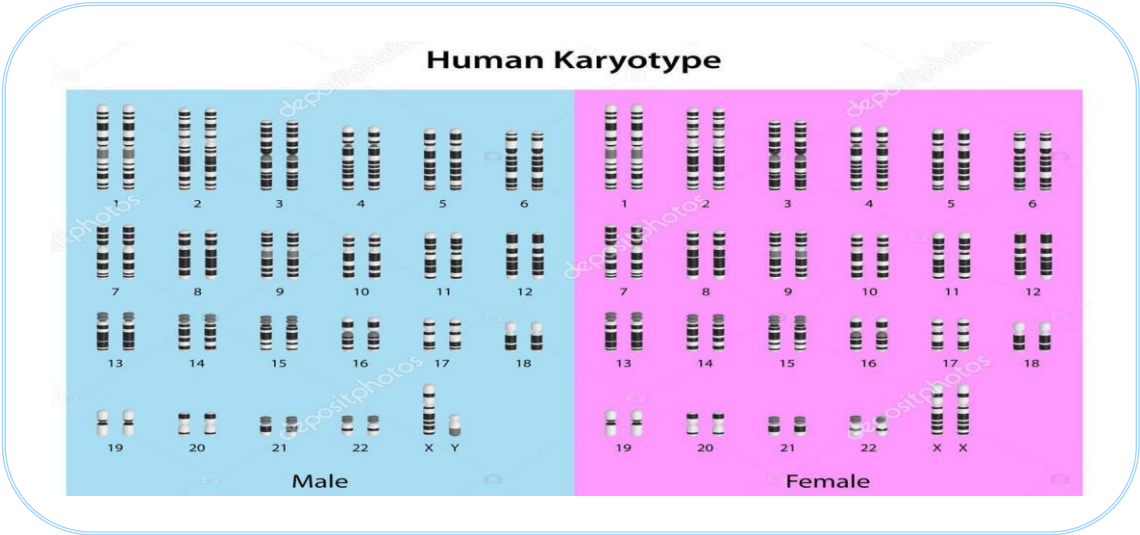


Figure 2-3 : The Human Karyotype (Alberts *et al*., 2015).

The Human Karyotype Contains 23 Pairs of Chromosomes, 22 Autosomes and 1 Pair Sex Chromosomes (Male has XY, Female has XX Sex Chromosomes).

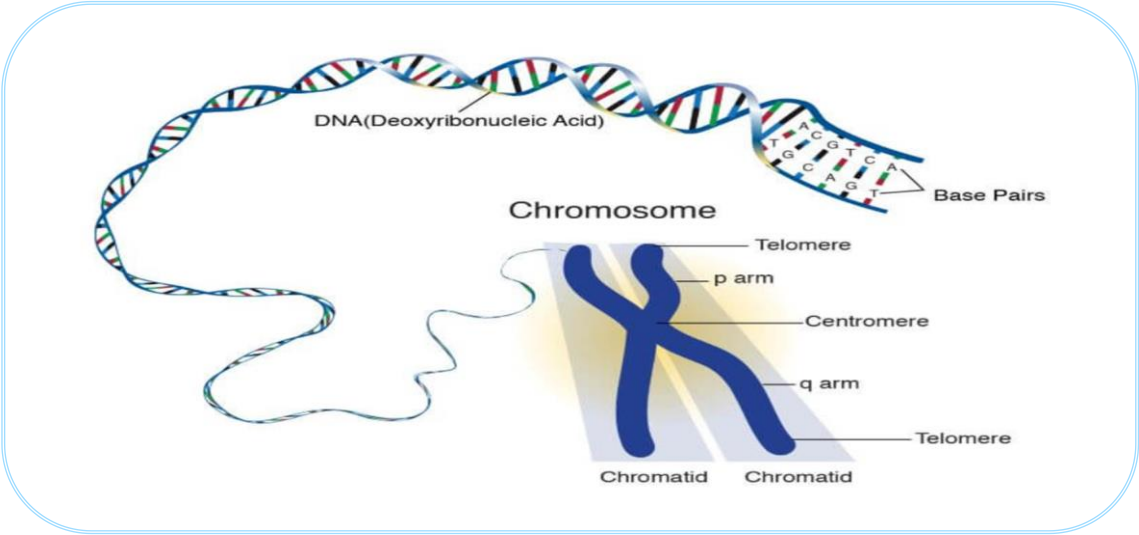


Figure 2-4 : Structure of Chromosome (Alberts *et al*., 2015).

2.4.2. Chromosome X

The two sex chromosomes (X and Y) found in human cells often define an individual's sex. Humans are bisexual animals; males have one X and one Y chromosome (XY), while females have two X chromosomes (XX) in their cells. The gene dosage in sex chromosomal biology is equal in male (XY) and female (XX) cells. The majority of X-linked genes are

found in two copies in female cells compared to one copy in male cells because the X chromosome contains many more genes than the Y chromosome. Consequently, one of the X chromosomes in female cells is rendered inactive in order to offset this imbalance in gene dosage (Souyris *et al*., 2019).

This XCI is a process that starts at the four cell stage of embryonic development and is passed down through somatic cell divisions throughout the course of a person's life (Souyris *et al*., 2019). Extremely interesting to note that the percentage of genes that escape inactivation varies significantly throughout the regions of the X chromosome. The majority of genes that do so are located in clusters in the short arm of the (Xp), which is home to the *TLR7* locus (Laffont and Guéry, 2019).

Approximately 1100 genes are found on the X chromosome; some of these genes are involved in innate or adaptive immunity (Libert *et al*., 2010). In the short arm of the X chromosome (Xp), the genes encoding the RNA-sensing endosomal *TLRs* (*TLR7* and *TLR8*) are located on a pseudoautosomal region (Souyris *et al*., 2019) as in Figure (2-5).

In most cases, females have one of the two female sex chromosomes inactivated (Lyon 1992; Schurz *et al.* 2019). *TLR7* genes, and perhaps *TLR8* as well, are immune cell genes that do not experience similar suppression in females. Consequently, both chromosomes' genetic material is expressed, while males only have one copy of the X chromosome (Groot and Bontrop, 2020).

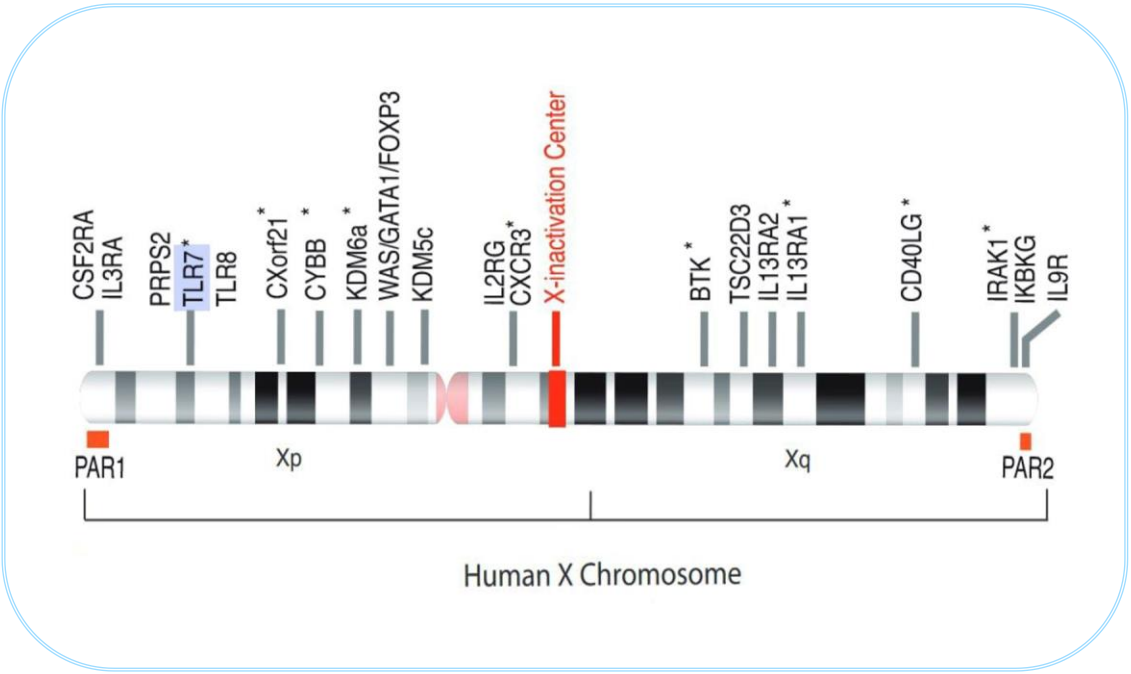


Figure 2-5 : The Human X Chromosome

The (Figure 2-5) shows the human X chromosome , the human *TLR7* gene, situated on the X chromosome's short arm (Youness *et al.*, 2021).

2.4.3. Toll Like Receptors

2.4.3.1. Toll-like Receptors (*TLRs*) Definition

Transmembrane glycoprotein receptors known as toll-like receptors (*TLRs*) are essential to the functioning of the innate immune system. They are the first pattern recognition receptors (PRRs) to be discovered and described in mammals, and they are typically found on the cell surface, with the exception of *TLR3*, *TLR7*, *TLR8*, and *TLR9* (Figure 2-6). *TLRs* may be able to prevent and cure cancer due to their function in the immune system (Semlali *et al.*, 2018) .

The family of proteins known as pattern recognition receptors (PRR), which includes toll-like receptors (*TLR*), is responsible for enhancing immunity through the identification of damage-associated molecular

patterns and pathogen-associated molecular patterns (PAMP) (Janku *et al*., 2022) . They detect endogenous and external danger signals as innate immune sensors using pattern recognition molecules (DAMPs and PAMPs). Through their signaling pathways, they also initiate an adaptive immune response by inducing several inflammatory cytokine genes and activating IRF3 and NF-κB (Figure 2-6). Several studies have demonstrated that *TLR* variants alter the cellular immune response, and that the incidence and severity of cancer, as well as susceptibility to inflammatory and infectious diseases, are all correlated with some of these variants (Semlali *et al*., 2018) .

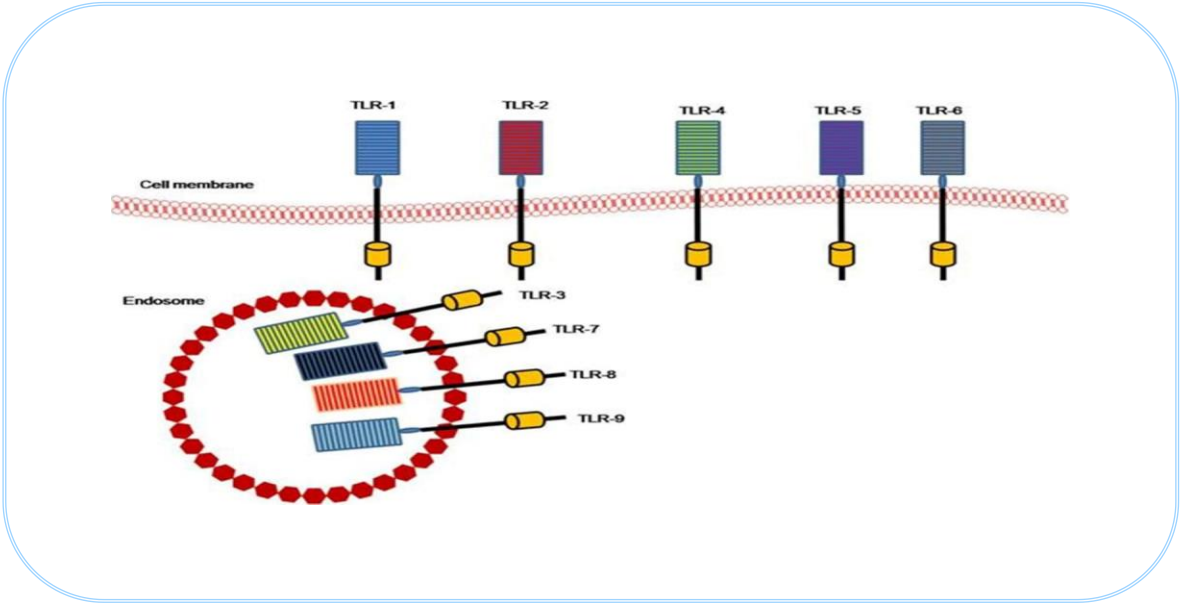


Figure 2-6 : Members of *TLR*s Family and Their Location (Semlali et al., 2018)

PAMPs, also known as pathogen-associated molecular patterns, are molecules that are commonly connected to illnesses. These compounds are recognized by proteins called Pattern Recognition Receptors (PRRs). PRRs are linked to extracellular forms that are present in the circulation and interstitial fluids, as well as subcellular compartments such as the cytosol, cellular and endosomal membranes, and the cytoplasm. *TLR*s represent one

of the four primary sub-groups of PRRs (Amarante-Mendes *et al*., 2018).

According to certain research on *TLRs* linked to cancer, *TLR* signaling can influence carcinogenesis through both pro- and anti-tumor effects, depending on the specific *TLR* and kind of cancer. Through immune cells or by directly targeting tumor cells to cause death, *TLR* activation strengthens the anti-tumor immune response (Urban-Wojciuk *et al*., 2019; Duan *et al*., 2022).

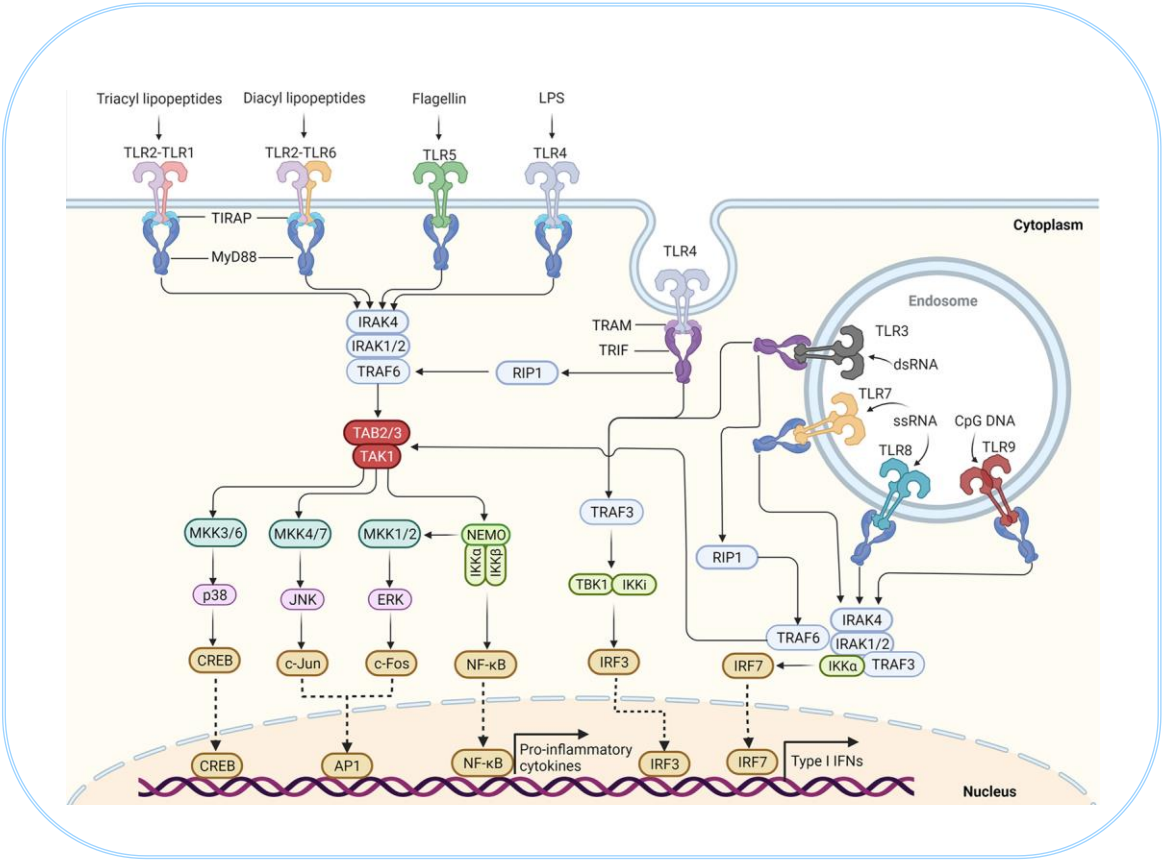


Figure 2-7 : *TLR* Signaling Pathway in Innate Immune Cells

Figure 2-7) showed the *TLR* signaling pathway in innate immune cells, where : *TLR5, TLR4,* TLR6, and heterodimers of *TLR2-TLR1* or *TLR2-TLR6*) identify pathogen membrane components (microbial membrane lipids, proteins, and lipoproteins) at the cell surface. Localized in endosomes, *TLR3, TLR7–TLR8,* and *TLR9* are able to identify nucleic acids from both foreign and host bacteria. *TLR4* is located at the plasma

membrane, but when it is activated, it is endocytosed into endosomes (Duan *et al*., 2022).

TLR agonists trigger cytokine responses that, through suppressing T-regulatory cells, boosting CD8 T-cell activity, and encouraging the development of myeloid-derived suppressor cells, polarize the tumor microenvironment and antitumor immunity. (Janku *et al*., 2022).

2.4.3.2. *TLR* Signaling Pathway in Innate Immune Cells :

TLRs are able to identify endogenous byproducts of cellular damage, also known as damage-associated molecular patterns (DAMPs), and foreign pathogen-associated molecular patterns (PAMPs). Each member of the *TLR* family has a distinct cadre of natural ligands, which include DAMPs and PAMPs.

Endosomal *TLRs* identify nucleic acids, while *TLRs* on the cell surface identify proteins, lipoproteins, and lipids found in microbial membranes. *TLR* signaling causes the recruitment of intracellular adaptor proteins, which in turn causes the activation of transcription factors like proinflammatory cytokines to be produced, nuclear factor κ-light-chain enhancers of activated B cells (NF-κB), interferon regulatory factors 3 (IRF3) and IRF7, activating protein-1 (AP-1), and others.

All *TLRs* except *TLR3* use the intracellular signaling adaptor myeloid differentiation primary response gene 88 (MyD88); *TLR3* substitutes TRIF for MyD88. Activated *TLRs* recruit MyD88 and the serine-threonine kinase interleukin-1 receptor-associated kinase (IRAK) family (IRAK1, IRAK2, and IRAK4). These proteins comprise the Myddosome. This complex is then recruited by the E3 ubiquitin ligase tumor necrosis factor (TNF) R-associated factor 6 (TRAF6), which in turn activates and stimulates transformation growth factor beta-activated kinase 1 (TAK1), NF-κB and

mitogen-activated protein kinase (MAPK) pathways, and produces pro-inflammatory cytokines like interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor alpha (TNFα), and others.Endosomal *TLRs*, including as *TLR3*, *TLR7*, *TLR8*, and *TLR9*, activate NF-κB and IFR7 through the Myddosome and TRAF6. Instead of using MyD88, *TLR3* uses the adaptor TIR-domain-co ntaining adaptor-inducing interferon-β (TRIF), while *TLR4* signals through pathways that are both MyD88- and TRIF-dependent. Brings in the IKK-related kinases IKKε and TANK-binding kinase 1 (TBK1), which activates IRF3 and promotes the synthesis of type I interferons (IFNs). Moreover, TRIF connects with TRAF6 to facilitate NF-κB activation (Aluri *et al*., 2021).

TLRs may be used to treat and prevent cancer because of their function in the immune system (Semlali *et al.*, 2018) . Research on *TLRs'* role in cancer cells and their correlation with tumor growth is becoming increasingly interesting. On the other hand, a variety of immune cells, as well as human cancer cells (gastric, lung, cervical, etc.), express the functionally active *TLRs* (Sato *et al*., 2009).

The overall pattern of *TLR* expression in tumor cells points to a critical role for *TLR*-mediated signaling in the genesis of cancer. *TLR* ligands can, on the one hand, inhibit the growth of tumors. *TLR* agonists, on the other hand, can strengthen cancerous cells' resistance to chemotherapy and help them survive (Shcheblyakov et al., 2010). Nevertheless, it is unclear how important it is for different cancer cells to express different *TLRs* (Semlali et al., 2017).

Tumor cells may express more than one *TLR* to identify different DAMPs in their surrounding environment. This could improve the biological mechanism via which *TLR* activation creates an environment

conducive to growth and survival. Moreover, tumor cells that have activated their *TLRs* produce more immunosuppressive cytokines, like transforming growth factor (TGF)-β and interleukin (IL-10), when their *TLRs* are ligated, indicating that tumor cells also use *TLR* activation to evade tumor immune monitoring. However, the notion that *TLRs* are cancer inhibitors is supported by a number of published studies (Semlali et al., 2018). Therefore, we need to conduct more research on the role and genetic diversity of *TLR*s in cancer.

2.4.4. Toll Like Receptor 7

In the endosomal membrane of macrophages, monocytes, lymphocytes, and plasmacytoid dendritic cells, *TLR7* is a type 1 transmembrane glycoprotein. *TLR7* is mostly expressed in antigen-presenting cells (APCs), which include B cells and plasmacytoid dendritic cells (pDCs). The *TLR7* gene, which is located on chromosome Xp22.2 in humans, encodes it. *TLR7* conformationally changes in response to ligand binding, which triggers the production of inflammatory cytokines, including interleukin (IL-1, IL-6) and type 1 IFNs, through NF-κB (Janku *et al*., 2022; Sun *et al*., 2022).

TLR7 activation promotes B-cell differentiation, boosting the generation of autoantibodies and the creation of immunological complexes. *TLR7* improves the immune response by inducing type I IFN and cytokine production in pDCs (Fillatreau *et al*., 2021). According to (Fillatreau *et al*., 2021) one sensor for single-stranded viral RNA found in the endosomal membrane of specialized immune cells such as monocytes, macrophages, and dendritic cells is the Toll-like receptor 7 (*TLR7*).

TLR7 stimulation in tumor-bearing hosts activates antitumoral immunity, which has been shown in multiple cancer models to improve disease prognosis (Spinetti *et al*., 2016).

Numerous studies have examined the diverse expression patterns and clinical implications of *TLR7* in various cancer types. *TLR7* is a potential target for immunotherapy, a novel diagnostic biomarker, and a predictor of tumor progression and prognosis. *TLRs* on T cells participate in the control of T cell activity, function as co-stimulatory molecules, and stimulate T cell immunity, even if the exact mechanism of action of *TLR7* in cancer immunotherapy is yet unknown. To enhance the therapy of tumors, *TLR* agonists can stimulate both innate and adaptive immune responses as well as T cell-mediated anticancer responses. Furthermore, as prospective therapies for tumor-targeted immunotherapy, *TLR7/8* agonists have been discovered (Sun *et al*., 2022).

According to one study on MDSCs isolated from breast cancer patients, repolarization and suppression of MDSCs in breast cancer patients is caused by inhibition of STAT3 and stimulation of the *TLR7* pathway in MDSCs. Targeting *TLR7* is a promising strategy for both antiviral and antineoplastic therapies, according to a study that looked at the amount of *TLR7* in the serum of patients with urinary bladder cancer (UBC). Through evaluations of *TLRs* in vitro and in vivo, the researchers found that they have agonist effects in activating anti-tumor immunity and identified their potential for immunotherapy (Chi *et al*., 2017; Safarzadeh *et al*., 2020).

TLR7 is known to play a role in both the pathogenesis of autoimmune illnesses and the regulation of antiviral immunity. It is capable of identifying viruses that contain single-strand RNA, including HIV, HCV(Heil *et al*., 2004) and SARS-Co-V-2 (Poulas *et al*., 2020). Moreover,

it's interesting to note that *TLR7* confers antiviral immunity by recognizing viral single-stranded RNA (ss-RNA), such as SARS-CoV2. Moreover, pro-inflammatory cytokines and chemokines such as IFN-alpha, IFN-beta, and IFN-lambda are released upon *TLR7* activation and have been demonstrated to assist in viral clearance and decreased replication (Birra *et al.*, 2020).

The expression of *TLR7* is genetically determined and varies among individuals. The X-linked recessive *TLR7* gene has loss of function variations, particularly missense harmful changes, which may be the cause of sickness sensitivity to viruses such as COVID-19 (Fallerini *et al*., 2021).

2.4.5. Toll Like Receptor 7 Gene Definition

The X-chromosome contains the *TLR7* gene, which has several variants that may be linked to human diseases, such as asthma, autoimmune thyroiditis, systemic lupus erythematosus (SLE), and the susceptibility to and progression of HIV-1 infection (Zhang *et al*., 2020). Because *TLR7* is found on the X chromosome, it is expression higher in women than in men. In women, one X chromosome is typically inactivated; however, some X chromosome genes, such as *TLR7*, may not be inactivated (Souyris *et al*., 2018).

Because *TLR7* is therefore doubly expressed in B cells, monocytes, and plasmacytoid dendritic cells (pDCs), the amount of *TLR7* in these cells is larger in women than in males. The study that looked at the amount of *TLR7* in patients' serum from urinary bladder cancer (UBC) and its relationship to certain clinicopathological and demographic traits is one of the investigations into the involvement of the *TLR7* gene in different cancers (Chi et al., 2017).

Additionally, the susceptibility role of four *TLR7* single nucleotide variants (SNPs) in urinary bladder cancer as well as the effect of SNP on *TLR7* level are investigated. These SNPs are rs179018, rs179019, rs179020, and rs179021. According to this study, UBC patients had an average level of *TLR7* that is noticeably lower than that of UTI patients or controls. Similarly, only the rs179019 C allele significantly increased uncorrected frequency in UBC males compared to control males, while the C allele maintained a significantly lower uncorrected frequency in UTI females compared to control females for the four SNPs that are investigated. While some SNPs had an impact on *TLR7* levels in the serum, rs179019 is found to have a significant impact in female UTI patients (Fillatreau *et al*., 2021). Furthermore, the study's results demonstrated the significance of the *TLR7* gene's intron region in determining the propensity and risk of developing UBC. Although there is little evidence to support SNP in this context, it should not be undervalued. Accordingly, it's possible that the *TLR7* gene has significant functional variants that affect both the susceptibility to cancer and the response to treatment (Al-Humairi *et al*., 2019).

The human genome has numerous single nucleotide variants that can be identified, opening up new avenues for study and enhancing our knowledge of the relationship between genotype and phenotype. Bioinformatics techniques are increasingly being used at the molecular level to predict disease-associated SNPs stand for single nucleotide variants (Gaaib, 2022). SNPs have different impacts according to where in the DNA they are located. Some are located in the non-coding section of the DNA, while others are in the coding regions. While many variants have little effect on cell function, other SNPs can influence how a given medicine is absorbed, which may act as a predisposing factor for certain diseases (Vallejos *et al*., 2019).

CHAPTER THREE

Materials and Methods

3. Subjects, Materials and Methods

3.1. Subjects

A case-control study was conducted at the College of Applied Medical Sciences/ University of Kerbala. Fifty patients with breast cancer were enrolled in this study and diagnosed with breast cancer by oncologists in AL-Imam AL-Hussein Center for Oncology and Hematology in Karbala City / Iraq during the period from November 2022 to May 2024. The age range of the patients was from 29 to 75 years. The control group was composed of 50 apparently healthy women. Patients and control subjects were randomly selected according to inclusion and exclusion criteria. Demographic information about the participants was also collected according to the questionnaire, as presented in Appendix (1). Blood samples were drawn from both breast cancer patients and healthy volunteers to be used for investigation.

3.1.1. Inclusion and Exclusion Criteria

3.1.1.1. Inclusion Criteria

The following inclusion criteria were followed to include patients and control groups in the current study:

1. Patients with breast cancer whom they were clinically diagnosed by physicians.
2. females only.
3. Healthy subjects who appeared healthy who matched the same age.

3.1.1.2. Exclusion Criteria

Include patients group who have undergone mastectomy were excluded.

3.1.2. Questionnaire

Information from both patients and control groups was collected, which includes name, age, family history, phone number, sample number (the number that identifies the volunteer), date of sample collocation, and other questions, as shown in Appendix (1).

3.1.3. Study Design

The study was designed as a Case-Control Study carried out from November 2022 to May 2024, as shown in Figure (3.1).

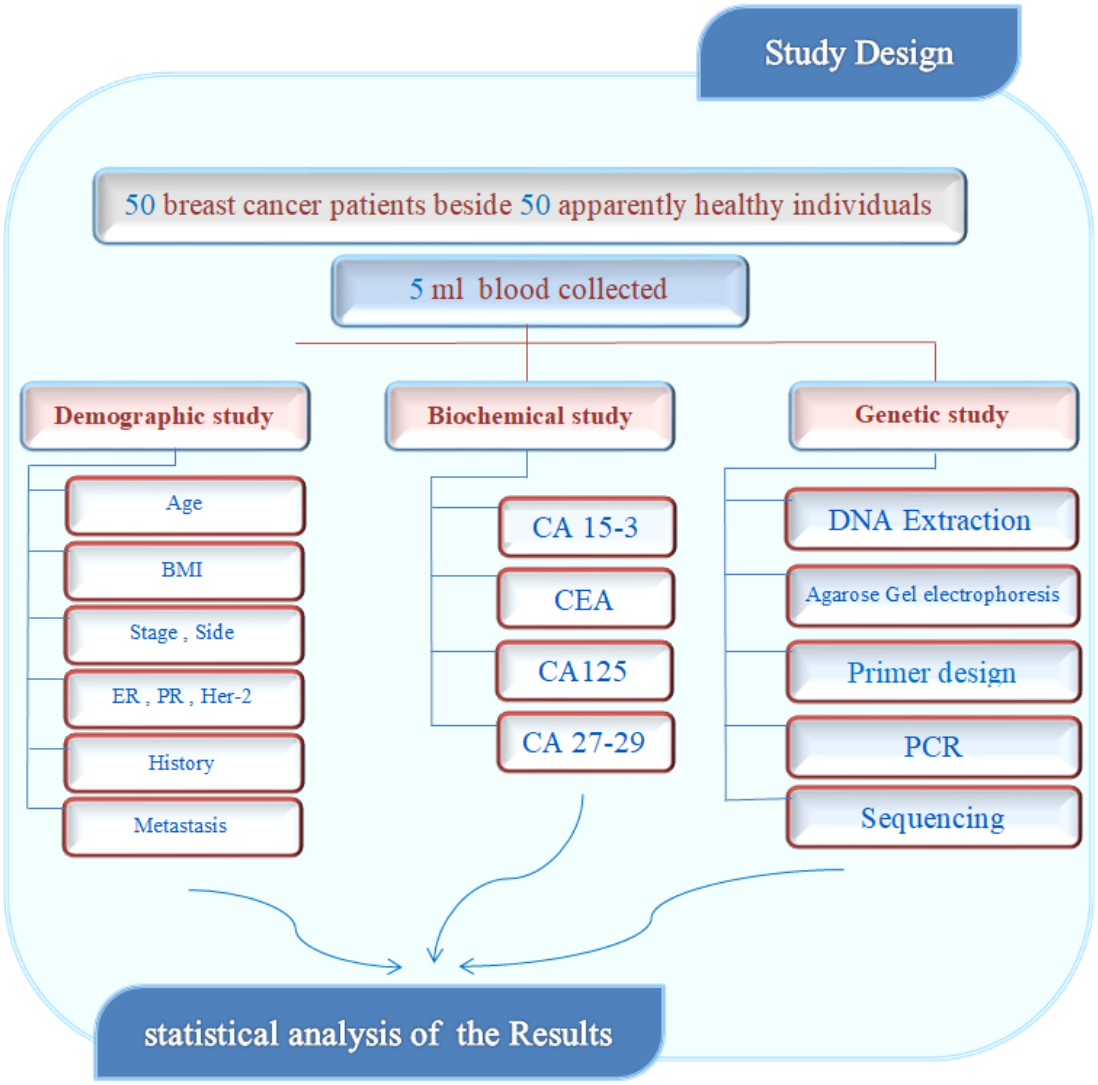


Figure (3-1): Study Design.

3.1.4. Ethical Approval

This study was authorized by the Institutional Ethics Committee at the College of Applied Medical Sciences, University of Kerbala, (IQ.UOK.CAMS.DCL.REC.1) and the Ethical Committee at Al-Imam Al-Hussein Center for Oncology and Hematology in Karbala. All subjects involved in this work were informed, and agreement was obtained verbally from each one before the samples' collection. Patient confidentiality was maintained throughout the research process.

3.2. Materials

3.2.1. Chemicals and Kits

In the current study, all the chemicals were used as provided without further purification; the chemicals were recorded in Table (3-1) and kits were recorded in Table (3-2).

Table 3-1: The Chemicals Used in the Current Study.

NO.	Chemicals	Origin	
		Company	Country
1	Agarose	Promega	USA
2	Blue loading Dye	Promega	USA
3	Distilled water		China
4	DNA leader 100 bp	Promega	USA
5	DNA Primers	Alpha DNA , S.E.N.C.	Canada
6	EasyTaq® PCR SuperMix	Promega	USA
7	Ethidium Bromide	Tokyo	Japan
8	Nuclease-Free water	Promega	USA
9	Red Safe Dye	Promega	USA
10	TBE Buffer 10X	Promega	USA

Table 3-2: The Kits Used in the Current Study.

NO.	Kits	Origin	
		Company	Country
1	A kit for extracting DNA	Promega	USA
2	Human Cancer Antigen 15-3 ELISA Kit (CA15-3)	BT LAB	China
3	Human Carcinoembryonic Antigen ELISA Kit (CEA)	BT LAB	China
4	Human Cancer Antigen 125 ELISA Kit (CA125)	BT LAB	China
5	Human Cancer Antigen 27-29 ELISA Kit (CA 27-29)	BT LAB	China

3.2.2. DNA Extraction Kit Components

ReliaPrep™ Blood gDNA Miniprep System and its components were demonstrated in Table (3-3). The system contains sufficient reagents for 100 isolations of genomic DNA from 200µl of whole blood samples.

Table 3-3: The Components of the DNA Extraction Kit.

NO.	Item	Quantity
1	Binding Buffer (BBA)	27.5 ml
2	Cell Lysis Buffer (CLD)	22 ml
3	Collection Tubes (2 ml)	400 / pk
4	Column Wash Solution (CWD)	165 ml
5	Nuclease-Free Water	25 ml
6	Proteinase K (PK) Solution	2.2 ml
7	ReliaPrep™ Binding Columns	100 / pk

3.2.3. Instruments and Equipments

Table (3-4): Demonstrates the Tools and the Instruments that were Used in this Study.

NO.	Item	country of origin
1	Abendorff Tube (2 ml)	China
2	Alcohol 70 % Ethanol	Iraq
3	Autoclave	Korea
4	Centrifuge Tube Rotator	USA
5	Deep Freeze	Japan
6	Disposable Face Masks	China
7	Distillery	GFL / Germany
8	Electronic Balance	Korea
9	Gel Electrophoreses System	UK
10	Gloves	China
11	Graduated Micropipettes (10),(100),(1000) µL	China
12	Incubator	Germany
13	K3-EDTA Blood Collection Tube	Henson medical/ chain
14	Laminar Flow Clean Bench (Laminar Flow Hood)	Lab Tic / Korea
15	Micro Centrifuge Capable of 14000×g (Eppendorff Centrifuge)	Hettich / Germany
16	Microwave System	LG / China
17	PCR (Thermal Cycler)	Clever / UK
18	Racks for (0.2 ml) PCR Tube	China
19	Racks for (1.5 ml) Eppendorf Tube	China
20	Serum Gel Blood Collection Tube	China
21	Sterile Syringe (5 ml)	China
22	Universal Pipette Tips (0-20 µL , 20 to 200 µL, or 200 to 1000 µL)	China
23	UV Transilluminator	UK
24	Vortex	Germany

3.3. Methods

3.3.1. Blood Samples Collection

Each participant provided five milliliters (ml) of venous blood, which was separated into two aliquots (2 ml in EDTA tubes and 3 ml in a Gel tube). blood was drawn using disposable syringes in a sterile condition. The EDTA tube collected blood was allowed to Freeze so that DNA could be

extracted from it later The blood collected in a gel tube was centrifuged to separate serum to be used later in biochemical tests (CA15-3, CEA, CA125, and CA27-29).

3.3.2. DNA Extraction

3.3.2.1. DNA Extraction Kit

ReliaPrep™ Blood gDNA Miniprep System was used for extracting DNA from blood samples.

3.3.2.2. DNA Extraction Procedure

1. The blood sample was completely thawed (blood samples were previously frozen), and then the blood sample was mixed well for at least 10 minutes in a rotisserie shaker at room temperature.
2. 20 µL of proteinase K (PK) solution was dispensed into a 1.5 mL microcentrifuge tube.
3. 200 µL of blood was added to the tube containing the proteinase K (PK) solution and mixed briefly.
4. 200 µL of cell lysis buffer (CLD) was added to the tube. Cover and mix by vortexing for at least 10 seconds. This spiral step was essential to get good yields.
5. Incubated at 56°C for 10 minutes.
6. During blood sample incubation, a ReliaPrep™ binding column was placed in an empty collection tube.
7. The tube has been removed from the heating block. 250 µL of binding buffer (BBA) was added, the tube was capped and mixed by vortexing for 10 seconds with a vortex mixer. It should be dark green at this point. , this spiral step is essential to getting good yields.
8. The contents of the tube were added to the ReliaPrep™ binding column, capped, and placed in a microcentrifuge.

9. Centrifuged for 1 minute at maximum speed. The binding column was checked to ensure that the lysate had completely passed through the membrane. If the membrane is still visible on top, centrifuge the column for another minute. It was ensured that the lysate had completely passed through the membrane.
10. The collection tube containing the flow was removed, and the liquid was disposed of as hazardous waste.
11. The connecting rod is placed in a new collection tube. 500 μL of column wash buffer (CWD) were added to the column, and centrifuged for 3 minutes at maximum speed. If any wash solution remained on the membrane, the column was centrifuged for another minute.
12. Step 11 was repeated twice for a total of three washes.
13. The column was placed in a new 1.5 ml microcentrifuge tube.
14. 100 μL of nuclease-free water was added to the column. Centrifuge for 1 minute at maximum speed.
15. The ReliaPrep™ binding post was eliminated, and the chamfer was retained.

    (No connecting rods or collecting tubes were reused).

3.3.3. Agarose Gel Electrophoresis

3.3.3.1. The Principle of DNA Electrophoreses

Agarose gel electrophoresis is the most effective way of isolation and recognition of DNA fragments. Using agarose gel electrophoresis, DNA was separated by loading it into pre-cast gel wells, dyeing them with Diamond Nucleic Acid Dye (safe dye), sometimes with Ethidium Bromide dye, and then applying a current. The phosphate of the DNA (and RNA) molecule is negatively charged, therefore when placed in an electric field,

DNA fragments will migrate from the negatively charged anode to the positively charged cathode. The rate of migration of a DNA molecule through a gel is determined by several factors, including the size of the DNA molecule; agarose concentration; voltage applied, and electrophoresis buffer (Lee *et al*., 2012).

3.3.3.2. Agarose Gel preparation

1. An amount of 1 gram of agarose was dissolved in 100 ml of 1X Tris-Borate-EDTA Buffer (TBE) to get 1% agarose gel.
2. Agarose was heated in the microwave until bubbles appeared and turbidity was cleared. It was left to be cold for about 20 minutes at room temperature (45-50 C°).
3. An amount of 3µl of Diamond Nucleic Acid dye (Red Safe dye) (10,000X) was added to the gel and mixed well. (The comb was placed in the gel tray at the appropriate location, and both of the tray's edges were sealed). After pouring the agarose solution into the horizontal tray, it was left to harden for about 30 to 45 minutes at room temperature.
4. The fixed comb was carefully removed and the gel tray is placed in the electrophoresis chamber.
5. TBE Buffer was added to the chamber until it reached 5 mm over the surface of the gel (Sanderson *et al*., 2014).

3.3.3.3. Loading and Running the Agarose Electrophoresis

1. An amount of 5µl of the DNA ladder (1 kilo base pair) was loaded to the first well of the agarose gel.
2. An amount of 3 µl of blue loading dye was mixed with 5 µl of each PCR product sample then loaded to its corresponding well of agarose gel.

3. The gel electrophoresis system was set at 100 volts for 60 minutes. Once finished, the gel was exhibited under a UV transilluminator, and photos were taken by the camera.

3.3.4. Primers' Reconstitution and Dilution

The Primer3plus bioinformatics tool was used to design the primers for this investigation. The primers were requested from Alpha DAN, S.E.N.C. in Montreal, Quebec, Canada, that was synthesized using standard phosphoramidite chemistry in automated computer-controlled synthesizers. The 2 ml microcentrifuge tubes containing the oligonucleotides were provided. Each oligonucleotide came with a detailed certificate that listed the amount (measured in OD260, micrograms, and picomoles), molecular weight, sequence, base content, and melting temperatures (three Tm were given, calculated by three different widely known methods) (Alpha DNA, S.E.N.C, 2023).

The oligonucleotides were shipped lyophilized (suitable for transportation at a wide range of temperatures, from below zero to +70 Celsius and even higher). Before opening the tube, the oligonucleotides were briefly centrifuged. To create a 100% stock solution, the manufacturer (Alpha DNA) instructed that the stock oligo be dissolved in Nuclease-Free Water at a certain volume (Alpha DNA, S.E.N.C, 2023).

The 100% stock solution of primers was kept at -20°C. A 0.5 ml Eppendorf tube containing 90 µl of nuclease-free water was filled with 10 µl of stock solution to provide a working solution of 10% for each primer. The working solution was also stored at - 20 C° until using.

3.3.5. Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is the most popular diagnostic method among molecular biological targeting nucleic acids(Lübeck and Hoorfar, 2003). Using two small, specifically designed fragments of DNA (primers or oligonucleotides), which serve as the two termini of the nucleic acid molecule to be amplified, the PCR allows the specific synthesis of a predetermined DNA region.

As long as primer-specific sequences correctly hybridize with complementary sequences on the target DNA molecule to be amplified, PCR amplification reactions are often highly specific. The forward and reverse primers control the PCR specificity as well as the length of the amplification products. The primer designated as upstream or forward is the one that hybridized closest to the (ATG) start codon of the gene (fragment) to be amplified, whereas the primer designated as downstream or reverse was the one that hybridizes closest to the (stop) codon (van Pelt-Verkuil *et al*., 2008).

3.3.5.1. PCR Components

In the present study, DNA fragments containing the targeted sequence of the *TLR7* gene, which was situated on chromosome X in the short arm, were amplified using traditional PCR. The PCR components included: DNA template (DNA samples), primers (Forward and Reverse), PCR master mix, and nuclease-free water (Table 3-1, Table 3-5).

3.3.5.2. Easy Tay PCR SuperMix

Easy Tay PCR SuperMix is a ready-to-use mixture of EasyTaq DNA Polymerase, dNTPs, and an optimized buffer. The SuperMir is provided at 2 concentrations and used at Ix concentration by adding a template, primers, and H_2O.

Table 3-5 : PCR Components.

NO.	Reagent	Volume
1	DNA sample	5 µl
2	Forward primer	2 µl
3	Reverse primer	2 µl
4	Master mix	8 µl
5	Nuclease free water	8 µl
Total volume		**25 µl**

3.3.5.3. PCR Product Detection.

Agarose gel electrophoresis was a technique used to separate PCR product bands. All PCR products were performed on 1% agarose gel and stained with fluorescent Red safe dye for analysis. The current study involves loading gel wells with 5µl of DNA ladder (100-1000 bp), 4µl of each PCR product mixed with 3µl loading dye (very carefully to avoid damaging the wells and cross-contaminating nearby wells), the gel electrophoresis system set to 70V for 60 minutes, and the gel displayed under a UV transilluminator.

Table 3-6 : The PCR Program (Primers : EXSON4).

Steps	Stage	Cycle	Step	Temp.	Time
Initial denaturation	1	1	1	94.0	4:00
Denaturation	2	35	1	94.0	0:30
Annealing			2	58.5	0:45
Extension			3	72.0	0:45
Final extension	3	1	1	72.0	5:00
Hold			2	4.0	HOLD

Table 3-7 : The PCR Program (Primers : INTRON2).

Steps	Stage	Cycle	Step	Temp.	Time
Initial denaturation	1	1	1	94.0	4:00
Denaturation	2	35	1	94.0	0:30
Annealing			2	57.0	0:45
Extension			3	72.0	0:30
Final extension	3	1	1	72.0	5:00
Hold			2	4.0	HOLD

3.3.5.4. The PCR Product Sequencing

In the current study, twenty (20) PCR product samples were sent to the Alpha DNA, (S.E.N.C.) corporation in (Montreal , Quebec , Canada), to perform nucleotide sequencing. Sanger sequencing method was applied by using an automated DNA sequencer. The results of sequencing were received by email, analyzed manually by using bioinformatics tools and aligned with human reference gene sequences already deposited on the National Center for Biotechnology Information (NCBI).

3.3.5.5. DNA Sequence Alignment

Alignment was the way of arranging the sequences of DNA to identify districts of similarity that may be a result of structural, functional, or evolutionary relationships among the sequences (Edgar and Batzoglou., 2006). The sequences of the 20 amplicons were sent to sequencing confirmed the specificity of the primer used in this study to target region on the *TLR*7 gene. The alignments were done by using the Basic Local Alignment Search Tool (BLAST) provided by NCBI.

The sequenced region of the target gene were analyzed and compared by using the bioinformatics tool Molecular Evolutionary Genetics Analysis X (MEGAX), in which the multiple alignments of the sequences were performed by the CLUSTALW program to confirm the presence of variants detected by the BLAST tool. The molecular location of all variants detected in the current study was registered and checked to identify the type and analyze variants and predict the functional consequences of variants by using tools provided by Ensemble Genome Browser. The identification of known and unknown, existing and novel of the variants detected in the current study was done by using tools of Ensemble Genome Browser.

3.4. Biochemical Markers Kits Used in the Current Study

3.4.1. Human Cancer antigen 15-3 (CA15-3) ELISA Kit

The purpose of this sandwich kit was precise quantitative detection of Human Cancer Antigen 15-3 (also known as CA15-3) in serum. The components of the kit were shown in the following Table.

Table 3-8 : CA15-3 ELISA Kit Components and Storage Conditions.

NO.	Components	Quantity (96T)	Quantity (48T)
1	Biotinylated Human CEA Antibody	1ml xl	1ml xl
2	Plate sealer	2 pic	2 pic
3	Pre-coated ELISA Plate	12* 8 well strips x1	12* 4 well strips xl
4	Standard Diluent	3ml xl	3ml x1
5	Standard Solution (1600 pg/ml)	0.5ml x1	0.5ml x1
6	Stop Solution	6ml x1	3ml x1
7	Streptavidin-HRP	6ml x1	3ml x1
8	Substrate Solution A	6ml x1	3ml x1
9	Substrate Solution B	6ml x1	3ml x1
10	User Instruction	1	1
11	Wash Buffer Concentrate (25x)	20ml x1	20ml x1
12	Zipper bag	1 pic	1 pic
Store the reagents at 2-8 C			

3.4.1.1. CA15-3 ELISA Kit Principles

An enzyme-linked immunosorbent assay (ELISA) was what this kit was for. Human CA15-3 antibody has been pre-coated on the plate. After being added to the sample, CA15-3 binds to the antibodies coated on the wells. Subsequently, the material was exposed to biotinylated Human CA15-3 Antibody, which binds to CA15-3. Next, the biotinylated CA15-3 antibody binds to streptavidin-HRP. During a washing phase, unbound

streptavidin-HRP was removed following incubation. The hue changes in direct proportion to the concentration of human CA15-3 once the substrate solution was added. The reaction was terminated by adding an acidic stop solution, and the absorbance was measured at W.L. 450 nm.

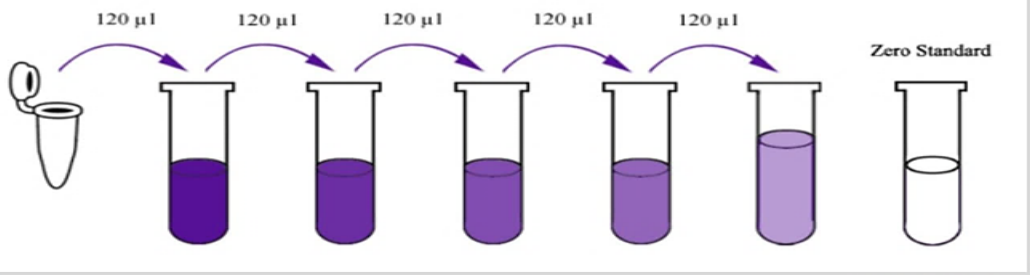
3.4.1.2. Reagent Preparation of CA15-3 ELISA Kit

All reagents were used at room temperature.

1. Standard

Normative To create a 2400/ml standard stock solution, reconstitute 120µl of the standard (480U/ml) with 120µl of standard diluent. Before producing dilutions, Allow the standard to settle, stirring gently, for fifteen minutes. By serially diluting the standard stock solution (240U/ml) to 120U/ml, 60U/ml, 30/ml, and 15/ml solutions using the standard diluent, duplicate standard points can be prepared. Standard diluent was the zero standard, or zero U/ml. Any leftover solution needs to be used within a month and frozen at -20°C. The recommended standard solution dilutions were as follows:

60U/ml	Standard No.5	120µl Original Standard+ 120ul Standard Diluent
240U/ml	Standard No.4	120µl Standard No.5+ 120µl Standard Diluent
120U/ml	Standard No.3	120µl Standard No.4+ 120µl Standard Diluent
30U/ml	Standard No.1	120µl Standard No.3+ 120µl Standard Diluent
15U/ml	Standard No.2	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
480U/ml	240U/ml	120U/ml	60U/ml	30U/ml	15U/ml

Figure (3-2): Dilution of Standard Solutions for (CA15-3)ELISA Kit.

2. Wash Buffer

Dilute the Wash Buffer (20 ml). 500 milliliters of 1x Wash Buffer can be obtained by concentrating 25 times in deionized or distilled water. If crystals have started to form in the concentrate, stir gently until all of the crystals have disappeared.

3.4.1.3. Assay Procedure of CA15-3 ELISA Kit

1. All samples, reagents, and standard solutions were made in accordance with the directions at room temperature.
2. The quantity of strips needed for the test was calculated. To use the strips, tires were put inside them. At 2–8°C, unused strips were kept.
3. The standard well was filled with 50 µl of standard.
4. The sample and standard wells (but not the blank control well) were filled with 40µl of the sample, 10µl of the anti-CA15-3 antibody, and 50µl of streptavidin-HRP. The mixture was then well mixed. The plate was coated with a sealant. The incubation period was 60 minutes at 37°C.
5. After removing the sealant, the plate was given five rounds of washing solution. Every wash, wells were submerged in 300ul of wash solution for 30 to 60 seconds. Each well was pipetted or decanted, and five washes with washing buffer were performed for instrumental washing. Paper towels or some type of absorbent material were used to wipe the plate.
6. Each well received 50 µL of substrate solution A and 50 µL of substrate solution B, respectively. The plate was incubated for ten minutes at 37 °C in the dark place and covered with fresh sealer.
7. The blue hue of each well-turned yellow the moment 50 µL of the stop solution was applied.
8. Using a microplate reader set at W.L. 450 nm, the optical density (OD

value) of each well was determined within 10 minutes of the stop solution being added.

3.4.2. Human Carcinoembryonic Antigen (CEA) ELISA Kit

The reliable quantitative detection of human carcinoembryonic antigen (CEA) in serum was the purpose of this sandwich kit. The components of the kit were shown in the following Table (3-9).

Table 3-9: CEA ELISA Kit Components and Storage Conditions.

NO.	Components	Quantity (96T)	Quantity (48T)
1	Biotinylated Human CEA Antibody	1ml xl	1ml xl
2	Plate sealer	2 pic	2 pic
3	Pre-coated ELISA Plate	12* 8 well strips x1	12* 4 well strips xl
4	Standard Diluent	3ml xl	3ml x1
5	Standard Solution (1600 pg/ml)	0.5ml x1	0.5ml x1
6	Stop Solution	6ml x1	3ml x1
7	Streptavidin-HRP	6ml x1	3ml x1
8	Substrate Solution A	6ml x1	3ml x1
9	Substrate Solution B	6ml x1	3ml x1
10	User Instruction	1	1
11	Wash Buffer Concentrate (25x)	20ml x1	20ml x1
12	Zipper bag	1 pic	1 pic
Store the reagents at 2-8 C			

3.4.2.1. CEA ELISA Kit Principles

This kit was intended for use in an ELISA test (enzyme-linked immunosorbent test). CEA antibody has been pre-coated on the plate. CEA binds to the antibodies coated on the wells after being added to the sample. Subsequently, the material was exposed to biotinylated Human CEA Antibody, which attaches itself to CEA. Next, the biotinylated CEA antibody binds to streptavidin HRP. During a washing phase, unbound streptavidin-HRP was removed following incubation. After adding the substrate solution, the color changes in direct proportion to the concentration of human CEA. The reaction was terminated by adding an acidic stop solution, and the absorbance was measured at W.L. 450 nm.

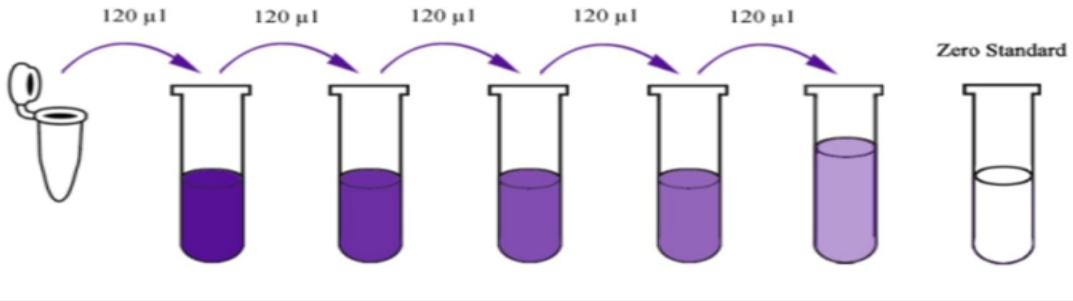
3.4.2.2. Reagent Preparation of CEA ELISA Kit

All reagents were used at room temperature.

1. **Standard**

Typical Reconstituted 120µl of the standard (1600 pg/ml) with 120µl of standard diluent yields 800 pg/ml standard stock solution. Let the standard sit for fifteen minutes with gentle stirring before making dilutions. To get 400 pg/ml, 200 pg/ml, 100 pg/ml, and 50 pg/ml solutions, the standard stock solution (800 pg/ml) has to be serially diluted with standard diluent. The zero standard, or zero U/ml, was the standard diluent. Any leftover solution needs to be used within a month and frozen at -20°C. The recommended dilutions for standard solutions were as follows:

800 pg/ml	Standard No.5	120µl Original Standard+ 120ul Standard Diluent
400 pg/ml	Standard No.4	120µl Standard No.5+ 120µl Standard Diluent
200 pg/ml	Standard No.3	120µl Standard No.4+ 120µl Standard Diluent
100 pg/ml	Standard No.1	120µl Standard No.3+ 120µl Standard Diluent
50 pg/ml	Standard No.2	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
1600 pg/ml	800 pg/ml	400 pg/ml	200 pg/ml	100 pg/ml	50 pg/ml

Figure (3-3) : Dilution of Standard Solutions for (CEA)ELISA Kit.

2. Wash Buffer

Dilute the Wash Buffer (20 ml). 500 milliliters of 1x Wash Buffer can be obtained by concentrating 25 times in deionized or distilled water. If crystals have started to form in the concentrate, stir gently until all of the crystals have disappeared.

3.4.2.3. Assay Procedure of CEA ELISA Kit

1. All samples, reagents, and standard solutions were made in accordance with the directions at room temperature.
2. The quantity of strips needed for the test was ascertained. To use the strips, tires were put inside them. At 2–8°C, unused strips were kept.
3. The standard well was filled with 50 µl of standard solution.
4. The mixture was carefully mixed after 40µl of the sample, 10µl of the anti-CA15-3 antibody, and 50µl of streptavidin-HRP were added to the sample and standard wells (but not the blank control well). The plate was coated with a sealant. The incubation period was 60 minutes at 37°C.
5. After removing the sealant, the plate was given five rounds of washing solution. Every wash, wells were submerged in 300ul of wash solution for 30 to 60 seconds. Each well was pipetted or decanted, and five washes with wash buffer were performed for instrumental washing. Paper towels or some type of absorbent material were used to wipe the plate.
6. Each well received the addition of 50 µL of substrate solution A and 50 µL of substrate solution B. The plate was incubated for ten minutes at 37 °C in the dark place while covered with fresh sealer.
7. Upon adding 50 microliters of the stop solution to every well, the blue hue instantly changed to yellow.

8. Within ten minutes of the stop solution being added, the optical density (OD value) of each well was ascertained using a microplate reader set at W.L. 450 nm.

3.4.3. Human Cancer antigen 125 (CA125) ELISA Kit

This sandwich kit was for the accurate quantitative detection of Human Cancer antigen 125(CA 125) in serum . The components of the kit were shown in the following Table (3-10).

Table 3-10: CA125 ELISA Kit Components and Storage Conditions.

NO.	Components	Quantity (96T)	Quantity (48T)
1	Biotinylated Human CA 125 Antibody	1ml xl	1ml xl
2	Plate sealer	2 pic	2 pic
3	Pre-coated ELISA Plate	12* 8 well strips x1	12* 4 well strips xl
4	Standard Diluent	3ml xl	3ml x1
5	Standard Solution (64U\L)	0.5ml x1	0.5ml x1
6	Stop Solution	6ml x1	3ml x1
7	Streptavidin-HRP	6ml x1	3ml x1
8	Substrate Solution A	6ml x1	3ml x1
9	Substrate Solution B	6ml x1	3ml x1
10	User Instruction	1	1
11	Wash Buffer Concentrate (25x)	20ml x1	20ml x1
12	Zipper bag	1 pic	1 pic
Store the reagents at 2-8 C			

3.4.3.1. CA125 ELISA Kit Principles

This kit was intended for use in an enzyme-linked immunosorbent test (ELISA). The plate has previously been pre-coated with the CA 125 antibody. The CA 125 in the sample binds to the coated antibodies on the wells once it was injected. Subsequently, the material was exposed to biotinylated Human CA 125 Antibody, which attaches itself to CA 125. Next, the biotinylated CA 125 antibody binds to streptavidin-HRP. During a washing phase, unbound streptavidin-HRP was removed following incubation. After adding the substrate solution, the color changes in direct

proportion to the concentration of human CA 125. The addition of an acidic stop solution ends the process, and the absorbance was measured at W.L. 450 nm.

3.4.3.2. Reagent Preparation of CA125 ELISA Kit

Before using, all reagents should be brought to room temperature.

1. **Standard**

Normative To create a 32 kU\L standard stock solution, reconstitute the 120µl of the standard (64 kU\L) with 120µl of standard diluent. Allow the standard to rest with moderate agitation for 15 minutes prior to making dilutions. Create duplicate standard points by serially diluting 16 kU\L, 8 kU\L, 4 kU\L, and 2 kU\L solutions from the standard stock solution (32 kU\L) at a ratio of 1:2 using standard diluent. The zero standard (0 kU/L) was standard diluent. Any leftover solution needs to be used within a month and frozen at -20°C. The recommended standard solution dilutions as in Figure (3-8).

2. **Wash Buffer** :

Dilute the Wash Buffer (20 ml). 500 milliliters of 1x Wash Buffer can be obtained by concentrating 25 times in deionized or distilled water. If crystals have started to form in the concentrate, stir gently until all of the crystals have disappeared.

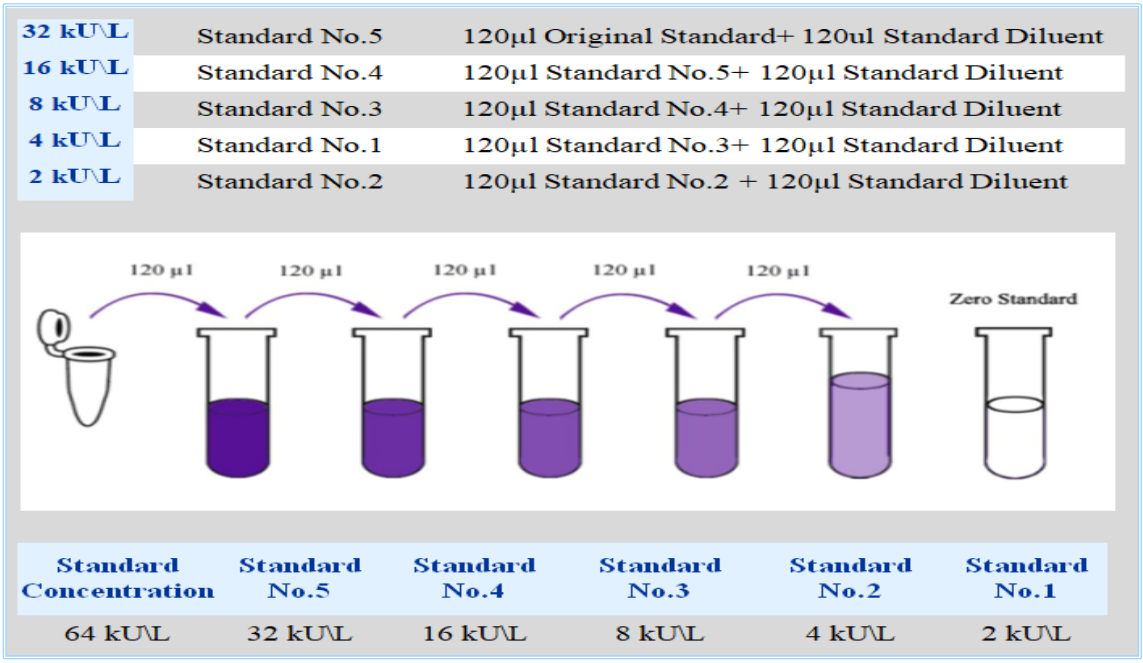


Figure (3-4) : Dilution of Standard Solutions for (CA125)ELISA Kit

3.4.3.3. Assay Procedure of CA125 ELISA Kit

1. All samples, reagents, and standard solutions were made in accordance with the directions at room temperature.
2. The quantity of strips needed for the test was ascertained. To use the strips, tires were put inside them. At 2–8°C, unused strips were kept.
3. The standard well was filled with 50 µl of standard.
4. After adding 40µl of the sample, 10µl of the anti-CA15-3 antibody, and 50µl of streptavidin-HRP to the sample and standard wells (but not the blank control well), the mixture was thoroughly mixed. A sealant was applied to the plate. At 37°C, incubation lasted for 60 minutes.
5. After removing the sealant, the plate was given five rounds of washing solution. Every wash, wells were submerged in 300ul of wash solution for 30 to 60 seconds. Each well was pipetted or decanted, and five washes with wash buffer were performed for instrumental washing. Paper towels or some type of absorbent material were used to wipe the plate.

6. Each well received 50 µL of substrate solution A and 50 µL of substrate solution B, respectively. The plate was incubated for ten minutes at 37 °C in the dark place while covered with fresh sealer.
7. The blue hue of each well-turned yellow the moment 50 µL of the stop solution was applied.
8. Within ten minutes of the stop solution being added, the optical density (OD value) of each well was ascertained using a microplate reader set at W.L. 450 nm.

3.4.4. Human Cancer Antigen 27-29 (CA27-29) ELISA Kit

This sandwich kit was for the accurate quantitative detection of Human Cancer antigen 27-29 (CA27-29) in serum . The components of the kit were shown in the following Table (3-12).

Table 3-11: CA27-29 ELISA Kit Components and Storage Conditions.

NO.	Components	Quantity (96T)	Quantity (48T)
1	Biotinylated Human CA 27-29 Antibody	1ml xl	1ml xl
2	Plate sealer	2 pic	2 pic
3	Pre-coated ELISA Plate	12* 8 well strips x1	12* 4 well strips xl
4	Standard Diluent	3ml xl	3ml x1
5	Standard Solution (160 U\ml)	0.5ml x1	0.5ml x1
6	Stop Solution	6ml x1	3ml x1
7	Streptavidin-HRP	6ml x1	3ml x1
8	Substrate Solution A	6ml x1	3ml x1
9	Substrate Solution B	6ml x1	3ml x1
10	User Instruction	1	1
11	Wash Buffer Concentrate (25x)	20ml x1	20ml x1
12	Zipper bag	1 pic	1 pic
Store the reagents at 2-8 C			

3.4.4.1. CA 27-29 ELISA Kit Principles

An enzyme-linked immunosorbent assay (ELISA) was what this kit was for. The CA 27-29 antibody has been pre-coated onto the plate. When introduced, the sample's CA 27–29 binds to the well-coated antibodies. Subsequently, the material was exposed to biotinylated Human CA 27-29 Antibody, which binds to CA 27-29.

Following the addition of streptavidin-HRP, the biotinylated CA 27-29 antibody attaches to it. During a washing phase, unbound streptavidin-HRP was removed following incubation. After adding the substrate solution, the hue changes in accordance to the concentration of human CA 27–29. The addition of an acidic stop solution ends the process, and the absorbance was measured at W.L. 450 nm.

3.4.4.2. Reagent Preparation of CA 27-29 ELISA Kit

1. Standard

All reagents were used at room temperature. Normative To create an 80 U\ml standard stock solution, reconstitute the 120µl of the standard (160 U\ml) with 120µl of standard diluent. Before producing dilutions, let the standard sit for 15 minutes with gentle agitation. To prep were duplicated standard points, dilute the standard stock solution (80 U/ml) in steps of 1:2 using standard diluent to get solutions of 40 U/ml, 20 U/ml, 10 U/ml, and 5 U/ml. The zero standard (0 U/ml) was the standard diluent. Any leftover solution needs to be used within a month and frozen at -20°C. The recommended standard solution dilutions as in Figure (3-6).

2. Wash Buffer

Dilute the Wash Buffer (20 ml). 500 milliliters of 1x Wash Buffer can be obtained by concentrating 25 times in deionized or distilled water.

If crystals have started to form in the concentrate, stir gently until all of the crystals have disappeared.

80 U\ml	Standard No.5	120µl Original Standard+ 120ul Standard Diluent
40 U\ml	Standard No.4	120µl Standard No.5+ 120µl Standard Diluent
20 U\ml	Standard No.3	120µl Standard No.4+ 120µl Standard Diluent
10 U\ml	Standard No.1	120µl Standard No.3+ 120µl Standard Diluent
5 U\ml	Standard No.2	120µl Standard No.2 + 120µl Standard Diluent

Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
160 U\ml	80 U\ml	40 U\ml	20 U\ml	10 U\ml	5 U\ml

Figure (3-5) : Dilution of Standard Solutions for (CA27-29) ELISA Kit.

3.4.4.3. Assay Procedure of CA27-29 ELISA Kit

1. All samples, reagents, and standard solutions were made in accordance with the directions. Before using, bring all of the reagents to room temperature. Room temperature was used for the examination.
2. The quantity of strips needed for the test was ascertained. To use the strips, tires were put inside them. At 2–8°C, unused strips were kept.
3. The standard well was filled with 50 µl of standard.
4. After adding 40µl of the sample, 10µl of the anti-CA 27-29 antibody, and 50µl of streptavidin-HRP to the sample wells and standard wells (but not the blank control well), the mixture was thoroughly mixed.

A sealant was applied to the plate. At 37°C, incubation lasted for 60 minutes.

5. After removing the sealant, the plate was given five rounds of washing solution. Every wash, wells were submerged in 300ul of wash solution for 30 to 60 seconds. Each well was pipetted or decanted, and five washes with wash buffer were performed for instrumental washing. Paper towels or some type of absorbent material were used to wipe the plate.
6. Each well received 50 µL of substrate solution A and 50 µL of substrate solution B, respectively. The plate was incubated for ten minutes at 37 °C in the dark place while covered with fresh sealer.
7. The blue hue of each well turned yellow the moment 50 µL of the stop solution was applied.
8. Within 10 minutes of the stop solution being added, the optical density (OD value) of each well was ascertained using a microplate reader set at W.L. 450 nm.

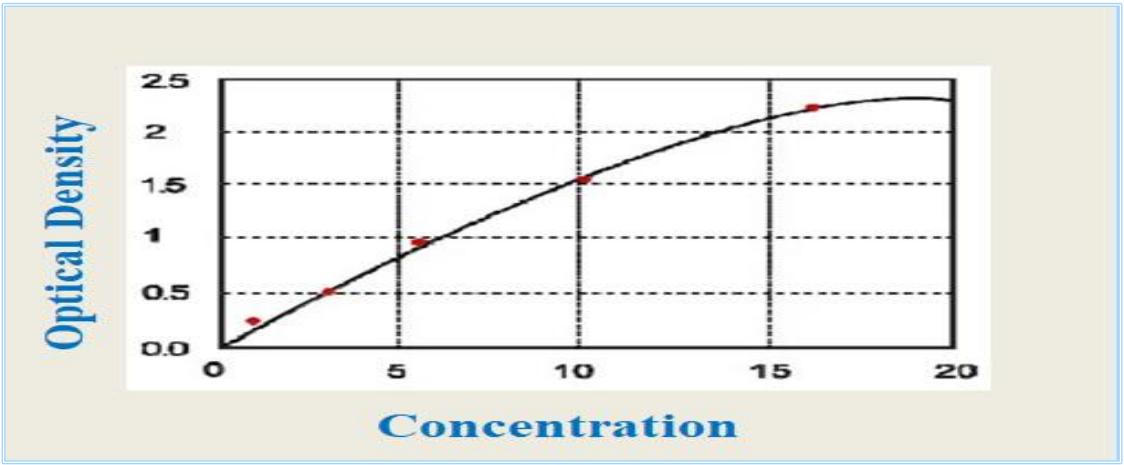


Figure (3-6): Sandard Curve for Biochemical Markers (CEA, CA 125, CA15-3, CA27-29) ELISA Kit.

3.5. Statistical Analysis

Statistical analysis was carried out using SPSS version 22.0 (SPSS, IBM Company, Chicago, IL, USA). Data was expressed as means ± standard error (SE) normally distributed and were compared using the independent samples T-test. Data was expressed as a median ± Interquartile range (IQR) if non-normal distributed and were compared using the Mann-Whitney U test. The P-values ≤ 0.05 were considered statistically significant differences.

3.6. Body Mass Index (BMI) Calculation

Body mass index or BMI is a statistical index using a person's weight and height to provide an estimate of body fat in males and females of any age. It is calculated by taking a person's weight, in kilograms, divided by their height, in meters squared, or BMI = weight (in kg)/ height^2 (in m^2).

The classifications for BMI according to the World Health Organization (WHO) are underweight, normal weight, overweight, or obese:

1. Underweight - BMI under 18.5 kg/m^2
2. Normal weight - BMI greater than or equal to 18.5 to 24.9 kg/m^2
3. Overweight – BMI greater than or equal to 25 to 29.9 kg/m^2
4. Obesity – BMI greater than or equal to 30 kg/m^2 :

    a. Obesity class I – BMI 30 to 34.9 kg/m^2
    b. Obesity class II – BMI 35 to 39.9 kg/m^2
    c. Obesity class III – BMI greater than or equal to 40 kg/m^2

(Hales et al., 2018; Weir and Jan ,2023).

CHAPTER FOUR

Results and Discussion

4. Results and Discussion

4.1. Demographic study

By collecting data from (50) patients with breast cancer according to a questionnaire form prepared for this purpose, to study the effect of some important factors on the incidence of the breast cancer in women, and these factors include Age, Weight, Height, Stage, Side of Breast, Progesterone Receptor, Estrogen Receptor, Her-2, Family History of Breast Cancer, Family History of other Cancers and Metastasis.

4.1.1. Age

In the current study, 100 women voluntarily enlisted, 50 women with breast cancer as a patient group, and 50 women apparently healthy as a control group. The range of age from 29-75 years. The mean age of the patient group was 49.49±1.48 and the control group was 47.94±1.51.

4.1.2. BMI

The results of the current study showed that the mean weight and height of the patient group were 76.35±1.76 and 157.7±0.85, respectively. The results also showed that the Body Mass Index (BMI) of the patient group appeared as three distinct categories represented by 14%, 22%, and 64% for the normal weight, overweight, and obese subjects, respectively (Figure 4-1).

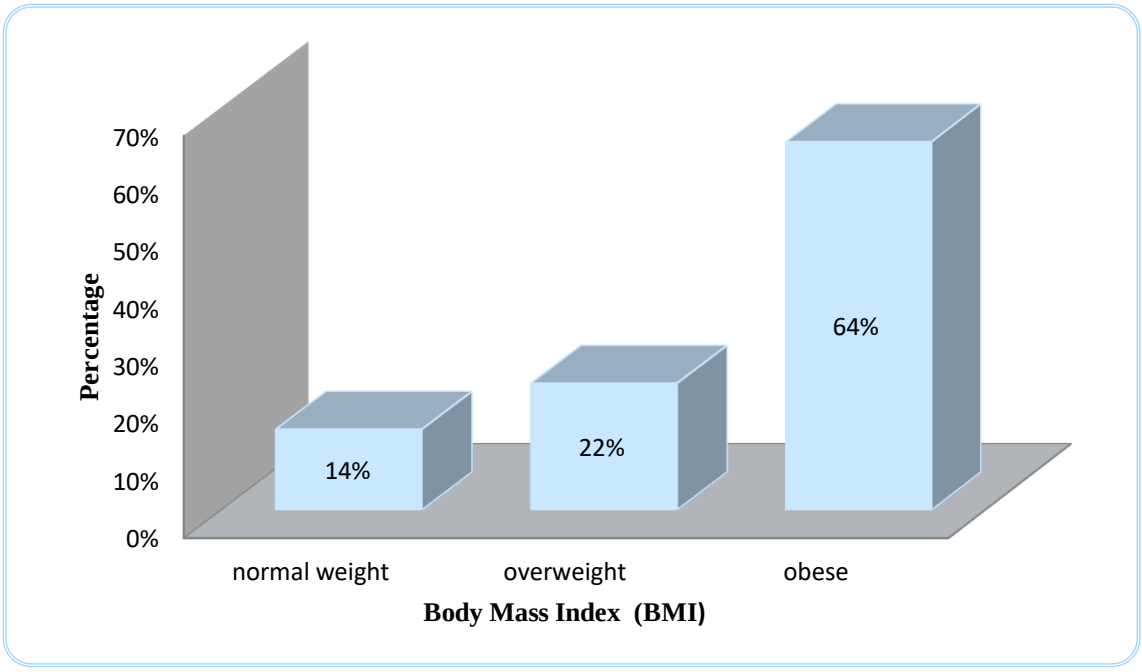


Figure (4-1): Distribution of Patient Group According to Body Mass Index (BMI).

4.1.3. Stage

The result of the current study indicated that the patient group showed an advanced staging rank of the breast cancer as 40 % of the participants were diagnosed at stage three while 60 % were allocated to stage four of breast cancer, the latter finding reflects late diagnosis perhaps due to a lack of follow up or complete unawareness (Figure 4-2).

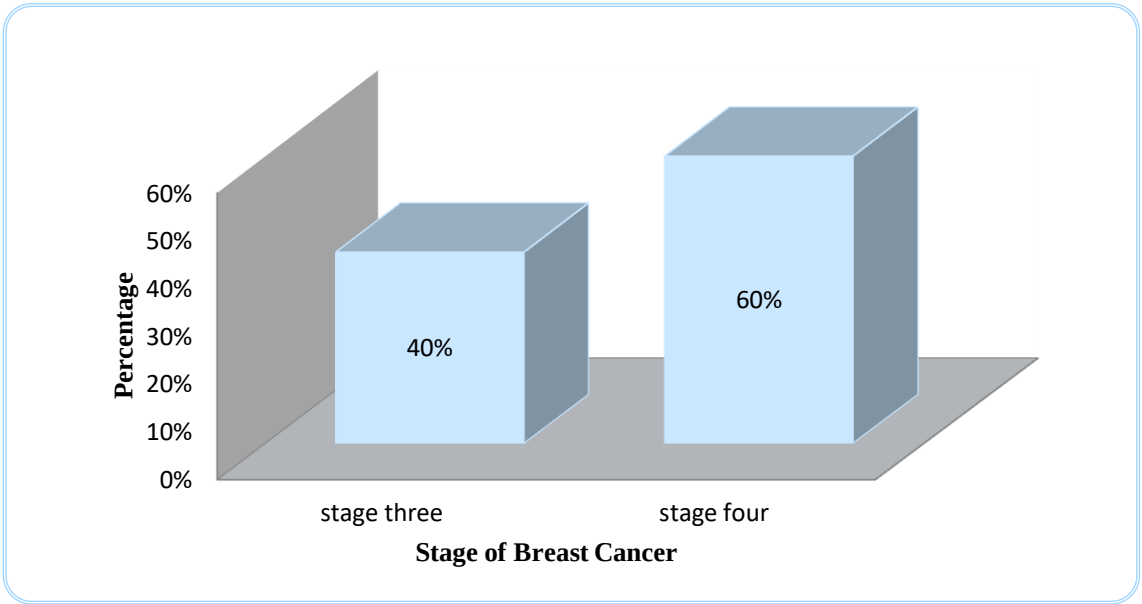


Figure (4-2): Distribution of Patient Group According to Stages of the Breast

Cancer.

4.1.4. Side of Breast (Left , Right)

The result of the current study showed that the breast cancer had initiated in 58 % of the affected women in the left breast while 42 % were primarily diagnosed as diseased in their right side breast (Figure 4-3).

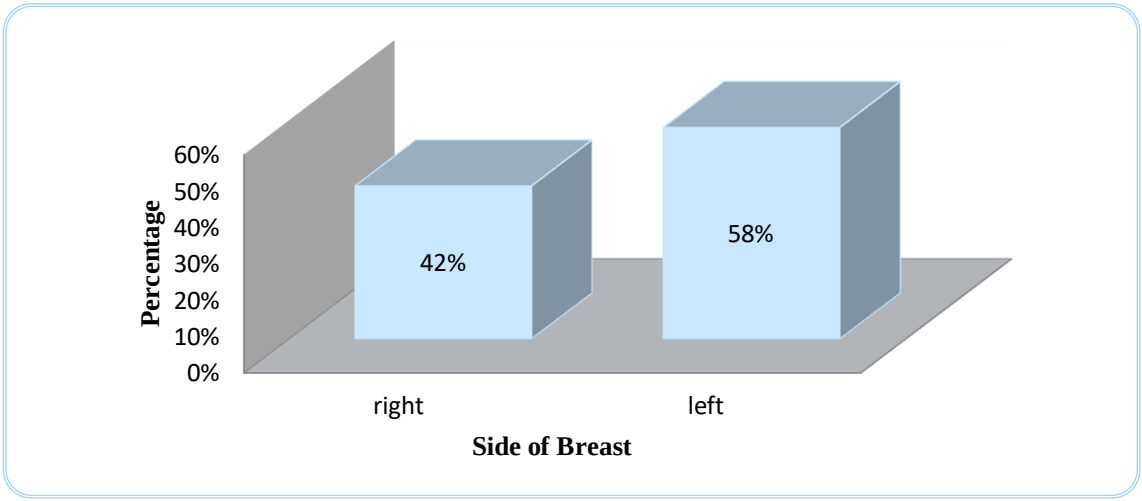


Figure (4-3): Distribution of Patient Group According to Side of the Breast.

4.1.5. Progesterone Receptor

Progesterone receptor (PR) expression of breast tissue was identified in 78% of the patients but the group of patients who did not express this marker was resembled by 22 % (Figure 4-4).

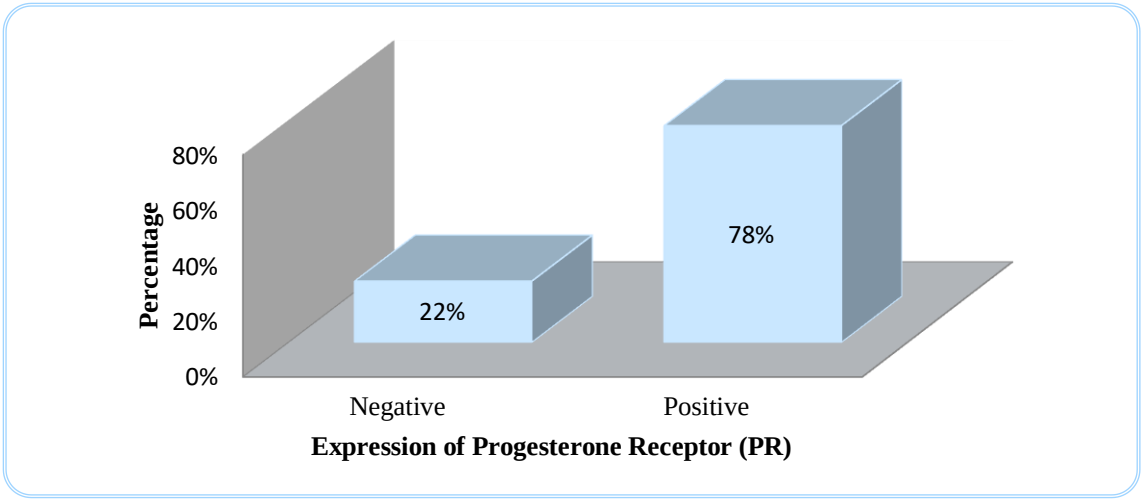
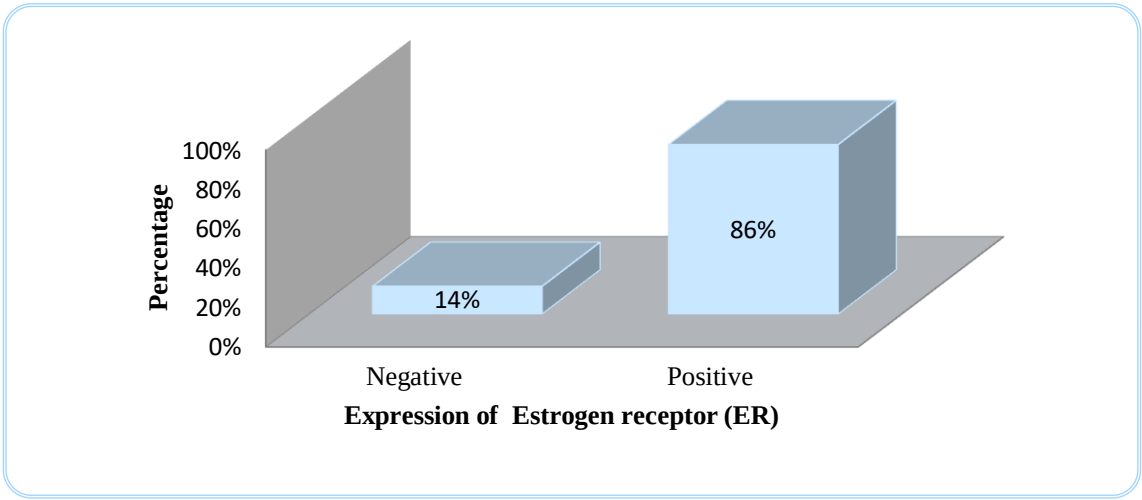


Figure (4-4): Distribution of Patient Group According to Progesterone Receptor (PR).

4.1.6. Estrogen Receptor

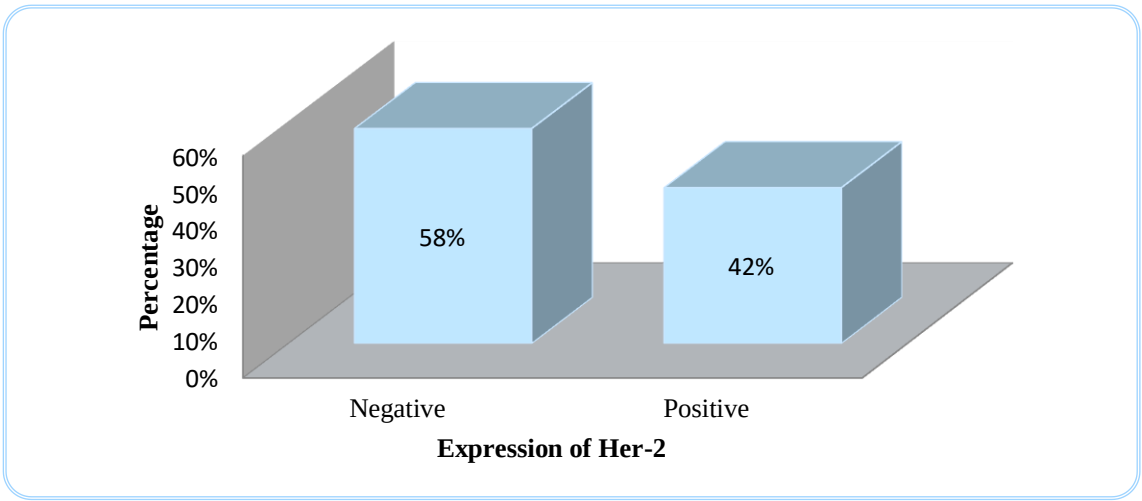
Estrogen Receptor (ER) expression was evidenced in 86 % of the patients while 14 % revealed negative results of the expression of this marker (Figure 4-5).



Figure(4-5): Distribution of Patient Group According to Estrogen Receptor (ER).

4.1.7. Human Epidermal Growth Factor Receptor 2 (Her-2)

On the contrary to the above-mentioned markers (PR and ER), Her-2 expression was not noticed in 58 % of the patients group. However, 42 % of the patients were positive for the expression of this marker (Figure 4-6).



Figure(4-6): Distribution of Patient Group According to Her-2.

4.1.8. Family History of Breast Cancer

Unexpectedly, the results of the current study showed no association between family history and breast cancer in the patient group under present study. 76 % of the newly diagnosed cases had no family history while 24 % showed such a manifestation (Figure 4-7).

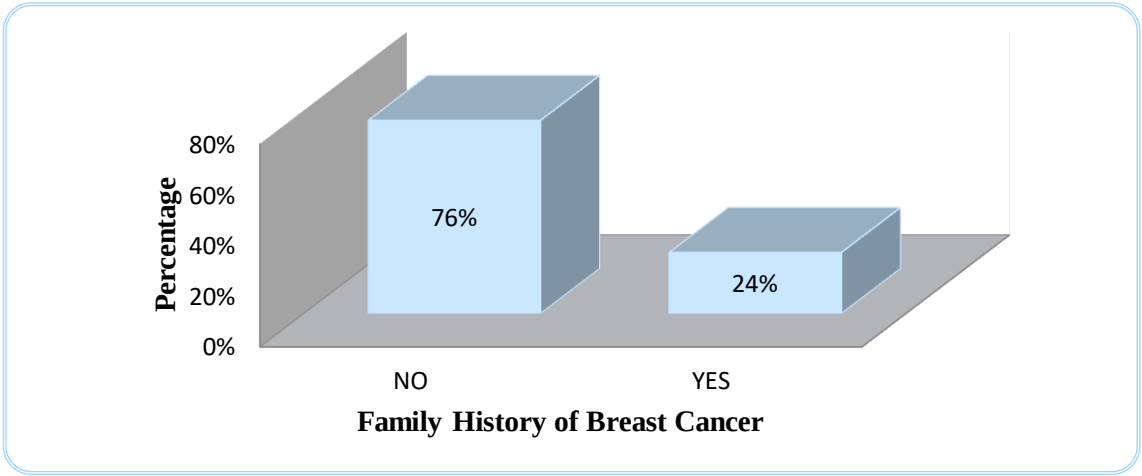


Figure (4-7): Distribution of Patient Group According to Family History of Breast Cancer.

4.1.9. Family History of other Cancers

results of this study showed a striking similarity for both the family history of breast cancer and the family history of other cancers were surveyed. It was clear that 70 % of cases had no previous family history of other cancers but 30 % of subjects had such an association (Figure 4-8) .

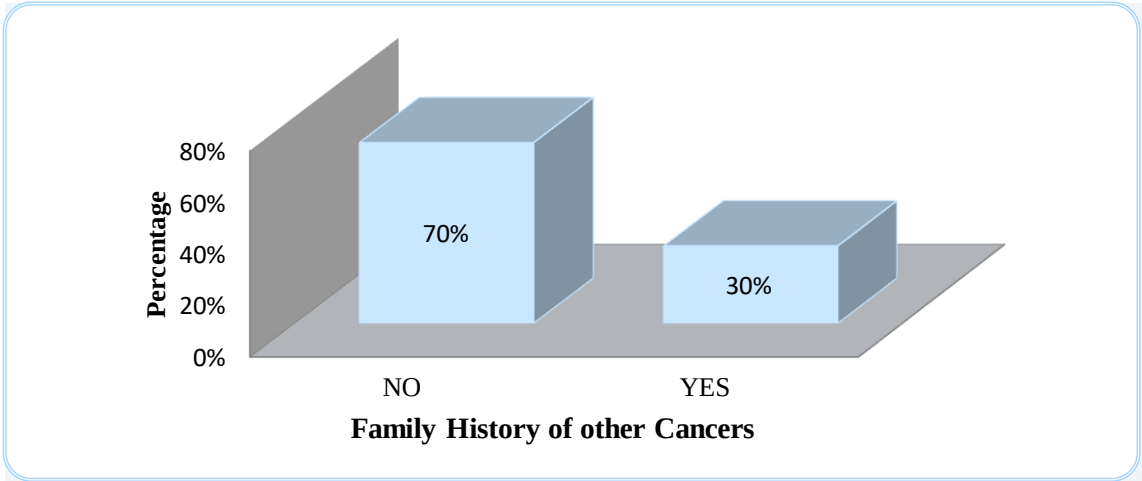
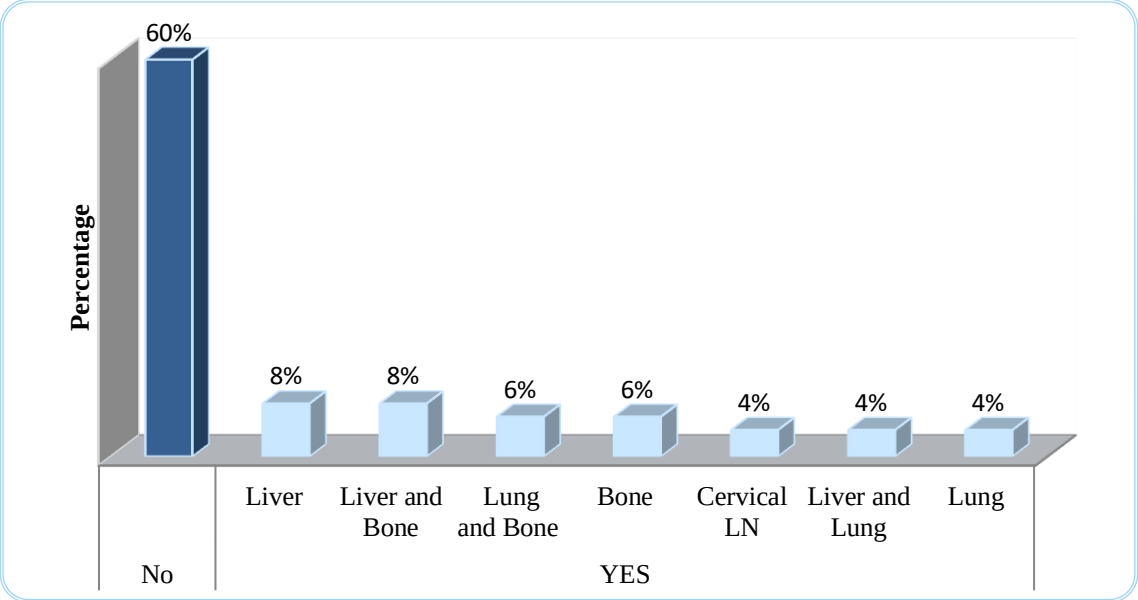


Figure (4-8): Distribution of Patient Group According to Family History of Other Cancer.

4.1.10. Metastasis

The (Figure 4-9) indicated the metastatic cases with the relevant percentages as per organ or system. It was obvious that 60 % of cases had no manifestations of metastasis while 40 % showed a metastatic activity distributed fairly evenly among different target tissues.



Figure(4-9): Distribution of Patient Group According to Metastasis state.

4.2. Biochemical Markers (CEA, CA125, CA15-3, CA27-29) Mean Value

Regarding the patients group, the results of the present study displayed that the CEA, CA125, CA15-3, and CA27-29 mean were 317.07±98.69, 21.85±15.63, 164.78±71.85, and 46.43±23.14 respectively. The results of the control group displayed that the CEA, CA125, CA15-3, and CA27-29 mean were 237.23±86.25, 13.05±5.34, 77.18±36.25, 28.72±8.18 respectively (Table 4-1).

Table 4-1: Description of Biochemical markers Value (n=50 patient and 50 control).

Tumor markers (normal value)	Mean ±SD	Min.	Max.	SE
Patient Groups				
CEA	317.07±98.69	115.963	1153.904	13.96
CA 125	21.85±15.63	7.337	52.817	2.21
CA 15-3	164.78±71.85	15.051	109.802	24.30
CA 27-29	46.43±23.14	11.617	107.06	3.27
Control Groups				
CEA	237.23±86.25	114.174	609.625	12.20
CA 125	13.05±5.34	6.353	38.336	0.75
CA 15-3	77.18±36.25	18.13	32.551	5.13
CA 27-29	28.72±8.18	11.264	39.725	1.16

The calculated Cohen's D (the effect size used to indicate the standardized difference between the two means) for the mentioned four comparisons of -0.861, -0.753, -0.705, and -1.021 suggests a large-sized effect, indicating that there was a practical and meaningful difference in the mean serum levels of the four tumor markers under study. The advantage was in favor of the patient group, highlighting it was potential significance for the differentiation between controls and patients (Table 4-2).

Table 4-2: Compared different chemical Markers in female patients with Breast cancer as Compared to the control group (n=100).

Tumor marker	Category	Frequency	Mean	Std. Error Mean	p-value	Cohen's D
CEA Pg/ml	Control	50	237.23	12.19	<0.001	-.861
	Patient	50	317.07	13.95		
CA 125 Ku/ml	Control	50	13.04	0.75	<0.001	-.753
	Patient	50	21.84	2.21		
CA 15-3 U/ml	Control	50	77.17	5.12	<0.001	-.705
	Patient	50	164.77	24.3		
CA 27-29 U/ml	Control	50	28.715	1.15	<0.001	-1.021
	Patient	50	46.42	3.27		

The mean of CEA was statistically significant across all groups. This result showed similarity with Zhao *et al.* (2021), who showed that preoperative serum CEA and CA15-3 levels were important factors affecting prognosis, in particular the recurrence. Lee *et al*. (2013) reported that a greater proportion of patients with preoperatively elevated tumor marker levels exhibited a more prominent elevation of tumor markers (CA15-3 and CEA) at recurrence compared to those without preoperatively elevated marker levels.

Regarding the relationship between marker levels and the metastatic site(s), the outcomes of earlier research have been contradictory. Nieder *et al*. (2017) reported that patients with lung, bone, or liver metastases had the highest proportions of elevated CEA levels and the highest proportions of elevated CA15-3 levels. Yerushalmi *et al*. (2012) found no significant differences in CA15-3 and CEA levels between different sites of metastasis. In contrast, a higher CEA level was noted independent of the location of the metastasis, while other studies showed a correlation between the rise of CA15-3 at recurrence and bone metastasis (Geng *et al.* 2012*).*

In addition, *(*Li, *et al*. ,2019) reported that the elevation of CA15-3 does not always lead to the diagnosis of metastatic disease. Benign conditions (infection, inflammation, and trauma) and other malignancies may give rise to increased marker concentrations, also reported that serum CA15-3 levels worked better as biomarkers for patients with lung cancer, breast cancer, ovarian cancer, nephritic syndrome, and type 2 diabetes, among many other cancers and noncancerous diseases. Therefore, the increased serum CA15-3 levels were not specifically associated with breast cancer .

The *MUC16* gene encodes CA125, which is a prognostic factor for various malignancies, particularly in gynecological oncology. However, it was still unclear how exactly CA125 affects breast cancer (Li *et al.* 2019). The mean of CA125 was statistically significant across all groups. This result showed similarity with result of (Charkhchi *et al.* ,2020), who stated that elevated levels of CA125 serve as an early indicator of this illness, and research continuously demonstrates that CA125 is the gold standard for tumor identification in ovarian cancer.

According to research by (Piatek *et al*., 2020), CA125 levels vary significantly during the disease's downstage, and an increase in CA125 levels in patients undergoing a remission period of time during (anticancer medication was used to either completely eradicate or significantly reduce the signs and symptoms of cancer) was a strong predictor of the disease's recurrence.

(Li, *et al.*, 2019), reported that the patients had a greater risk of lung and bone metastases if their CA125 values were higher. Greater CA125 has been linked to a better prognosis in individuals with stage IV or recurrent breast cancer, despite the fact that it was not predictive in all cases of breast cancer. The results of this study conflicted with those published by (Opstal *et al.*, 2012), who claimed that CA125 was not a reliable biomarker for breast cancer patient diagnosis or grading and may even be unnecessary when combined with additional biomarkers like CA15-3.

These findings were consistent with the data reported by (Funston *et al.*, 2020), their results imply that CA125 may be regarded as a disease marker that was least effective in identifying the disease when it was benign and can identify the disease at an intermediate degree of severity. Therefore, CA125 by itself cannot serve as a perfect biomarker for every type of cancer.

Another study also found that using CA125 alone was insufficient to predict the prognosis of cancer and instead focused on using numerous markers to improve prognostic accuracy (Chen *et al.* 2018).

For all groups, the mean of CA27-29 was statistically significant. This result was consistent with that of (Goodwin *et al.*, 2021), who verified that CA27-29, also referred to as (mucin 1), was another that has gained significance recently. They also noted that overexpression of *MUC-1* has been observed in breast cancer cells, especially in the advanced stages of the disease. In individuals with breast cancer, increased blood levels of CA27-29 have been linked to tumor burden and metastatic dissemination.

Additionally, a different study by (Hepp *et al.*, 2016) suggested that CA27-29 levels might be helpful for tracking the course of the disease in metastatic breast cancer (MBC) patients. It also demonstrated that an increase in CA27-29 levels might occur before radiological signs of the disease progress, indicating that it might act as a precursor to treatment failure or tumor recurrence. In this sense, it has been discovered that in patients with MBC, CA 27-29 levels were correlated with therapy response.

For instance, a drop in CA 27-29 levels following treatment would point to a positive outcome, whereas levels that were consistently high or growing might point to resistance or a poor response to therapy. (Rack *et al.*, 2016) discussed the routine use of CA27-29 as a clinical biomarker for breast cancer. They found that CA27-29 performs clinically similarly to CA15-3 and was useful for early recurrence detection in patients with advanced breast cancer who were being followed up on. This contradicts findings published by (Vaidyanathan *et al.*, 2012), who discovered the limitations of CA27-29 as a serum tumor biomarker similar to CA15-3.

Specifically, insufficient precision ought to be taken into account because people who have malignancies may have a high CA27-29 other than liver, lung, or colon cancers. Patients with benign liver, renal, and breast diseases also have higher rates of it. Further, it was increased in patients with ovarian cysts. Another study (Van et al., 2015) reported That, unlike CA15-3, CA27-29 has not been suggested as a biomarker for breast cancer.

Serological markers provide a non-invasive way to track MBC, although as stand-alone markers, they still have limited diagnostic and prognostic value. False-positive or false-negative findings may arise from these markers' limited sensitivity and specificity. Their clinical value may be increased by combining several markers or adding them to a panel together with other diagnostic techniques like imaging and histology (Pekarek *et al.*, 2023). These results were summarized in Table (4-8), Figure (4-19) for exon; Table(4-9), Figure (4-20) for intron region.

4.3. Results of Genetic Analysis

4.3.1. The DNA Extraction

ReliaPrep™ Blood gDNA Miniprep System DNA Extraction Kit successfully extracted DNA from the blood of participants. The presence of extracted DNA was detected in the1% agarose gel electrophoresis. Stained with red safe dye and sometime with Ethidium Bromide, and exposed to UV light as shown in Figure (4-10).

4.3.2. DNA Concentration and Purity

The DNA concentration was measured by using a Nanodrop Spectrophotometer to determine the quantity and purity of DNA. The absorbance (turbidity) of DNA molecules in the samples at 260 and 280 nm

wavelength are measured. The ratio of absorbance (A260/A280 nm) is used to estimate the purity of DNA. The ratio of ~1.8 is acceptable in general as "pure" for DNA (Tiwari et al.,2017).

4.3.3. Reconstitution and Dilution of Primers

The Primer3plus bioinformatics program (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was utilized to design the primers for this investigation. The primers were requested from Alpha DAN, S.E.N.C. in (Montreal, Quebec, Canada), and were synthesized on automated computer-controlled synthesizers utilizing standard phosphoramidite chemistry (Figure 4-11, Figure 4-12).

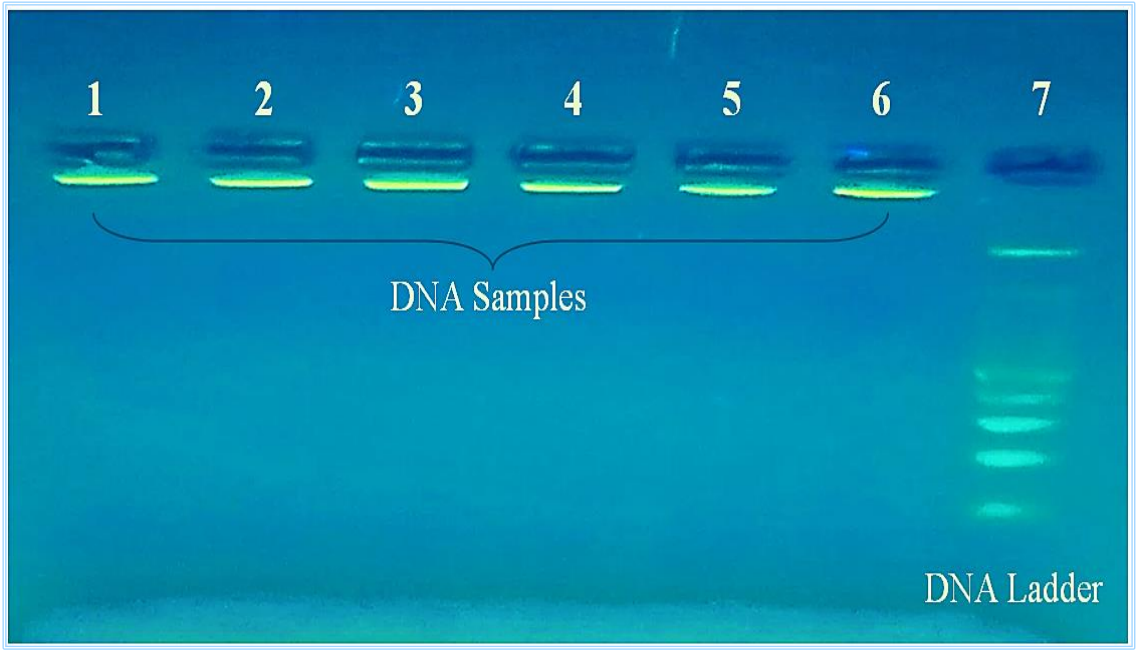


Figure (4-10): DNA Extraction Result from Blood Samples.

The presence of Extracted DNA Detected in 1% Agarose Gel Electrophoresis After Staining with Red Safe Dye and Exposed to UV Light (Figure 4-10**)**.

4.3.3.1. Intron2 Sequence of *TLR7* Gene in Primer 3 Plus Program

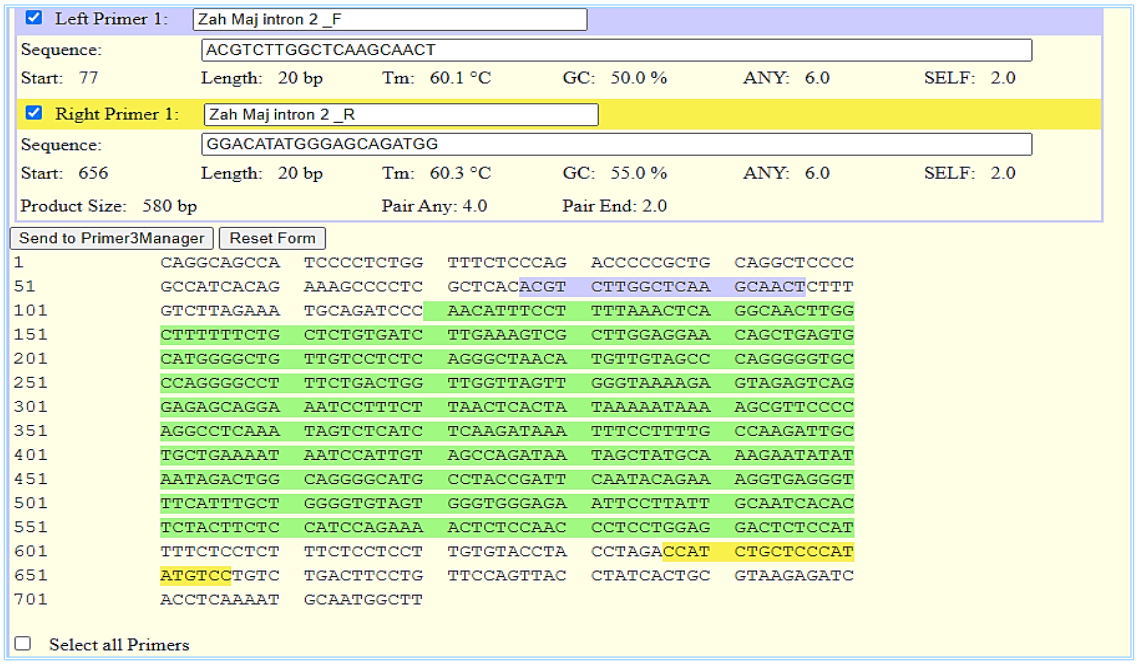


Figure (4-11): Forward and Reverse Primer Design for Intron2 *TLR7* Gene in Primer 3 Plus Program.

4.3.3.2. Exon4 Sequence of *TLR7* Gene in Primer 3 Plus Program

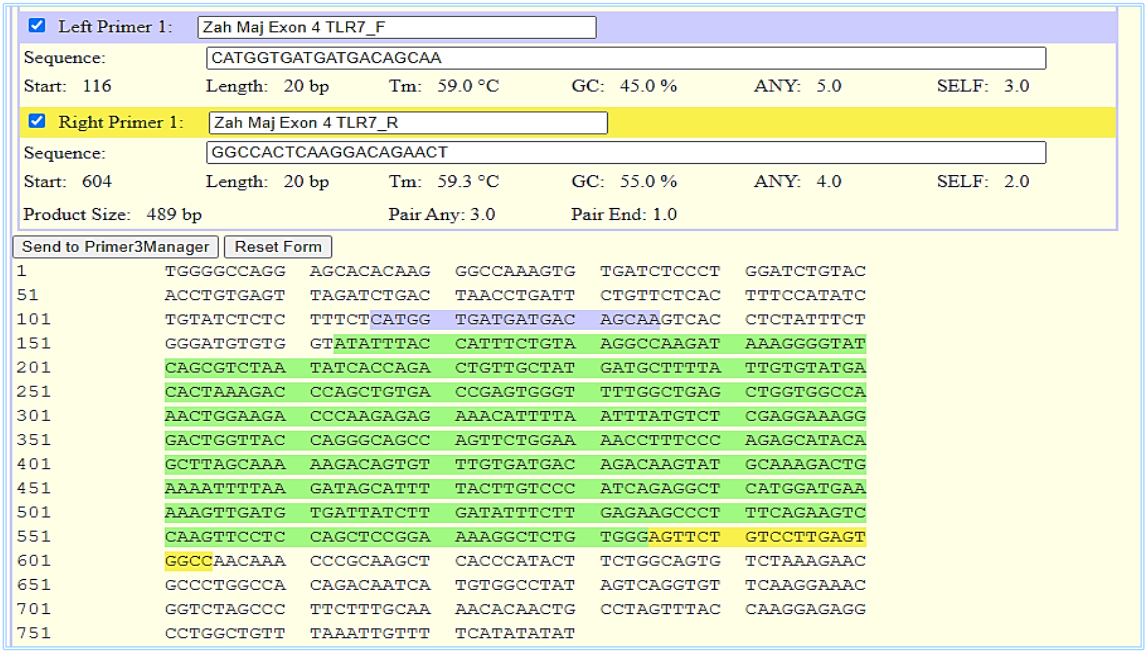


Figure (4-12): Forward and Reverse Primer Design for Exon4 *TLR7* Gene in Primer 3 Plus Program.

4.4. Polymerase Chain Reaction (PCR) Results

In this study, a DNA fragment including the target regions of the *TLR7* gene the Intron2 region (580 base pair) and the Exon4 region (489 bp), was amplified using traditional PCR. Every research sample had the PCR product, indicating that the target area of the *TLR7* gene was present (Figure 4-13, Figure 4-14).

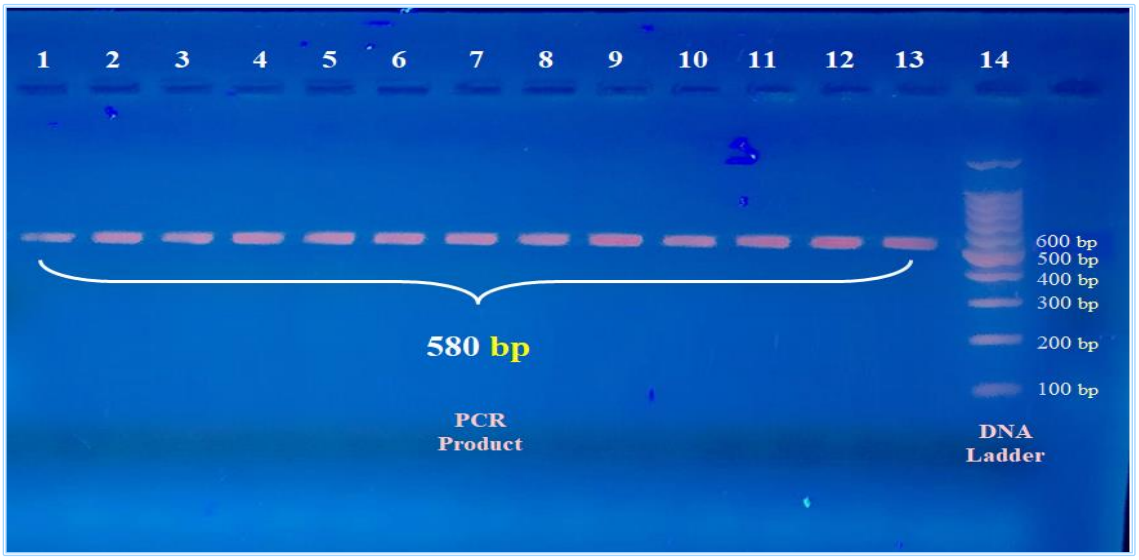


Figure (4-13): PCR Products (580 base pair) Detected in Study Samples by Gel electrophoresis at 100 volts for 60 minutes Indicates the Presence of the target region (Intron2) in the *TLR7* Gene (1-13 PCR Product and 14 DNA Ladder).

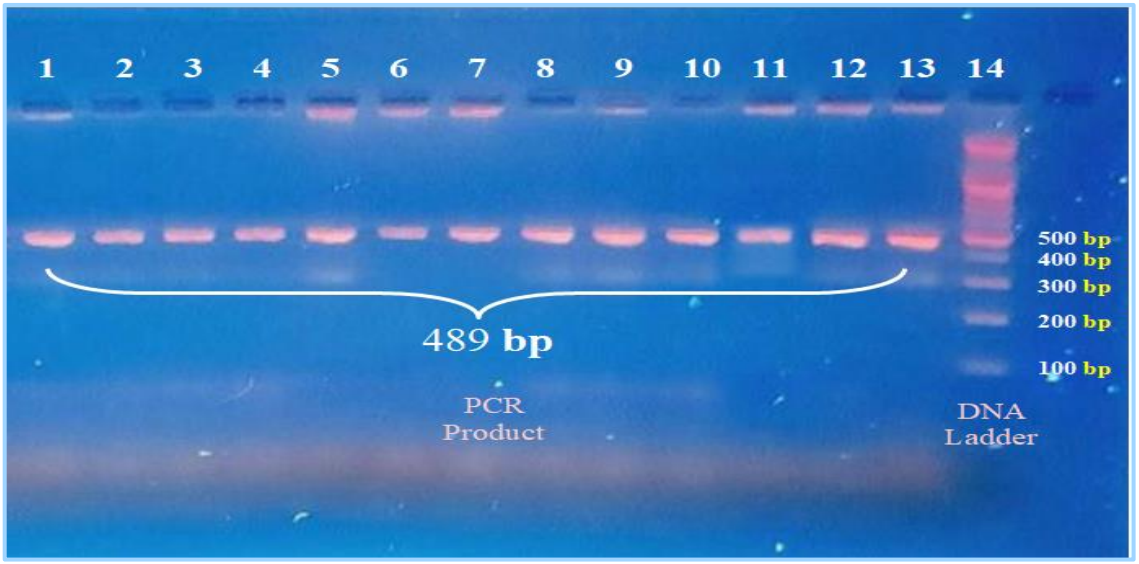


Figure (4-14): PCR Products (489 base pair) Detected in Study Samples by Gel electrophoresis at 100 volts for 60 minutes Indicates the Presence of the target region (Exon4) in the *TLR7* Gen, (1-13 PCR Product and 14 DNA Ladder).

4.4.1. PCR Product Sequencing

In the present study, 20 samples of the PCR products for the patient group were sent to the Alpha DNA, (S.E.N.C.) corporation in Montreal, Quebec, Canada, to perform nucleotide sequencing. A DNA sequencer that was automated was used to apply the Sanger sequencing procedure (Figure 4-15).

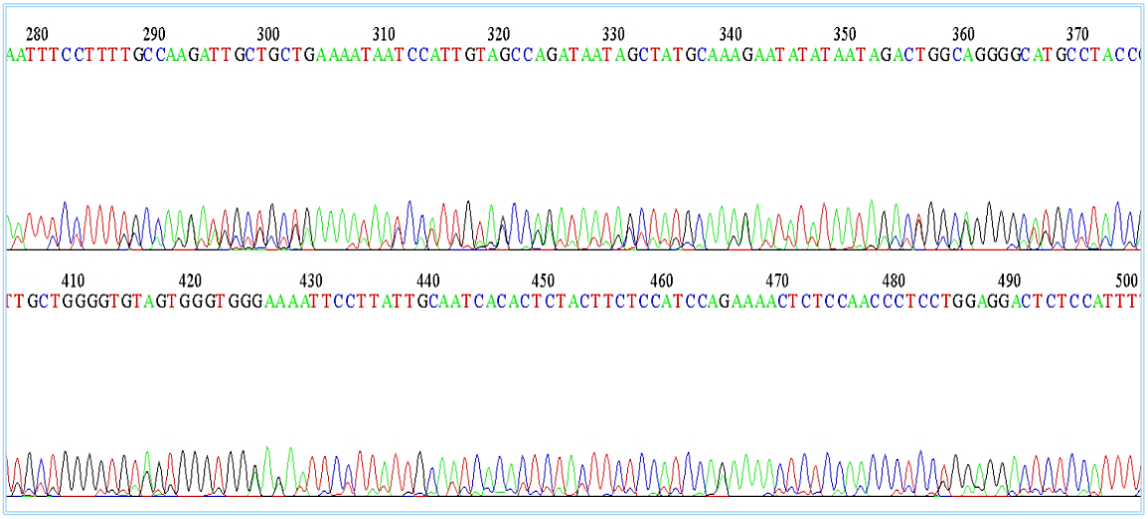
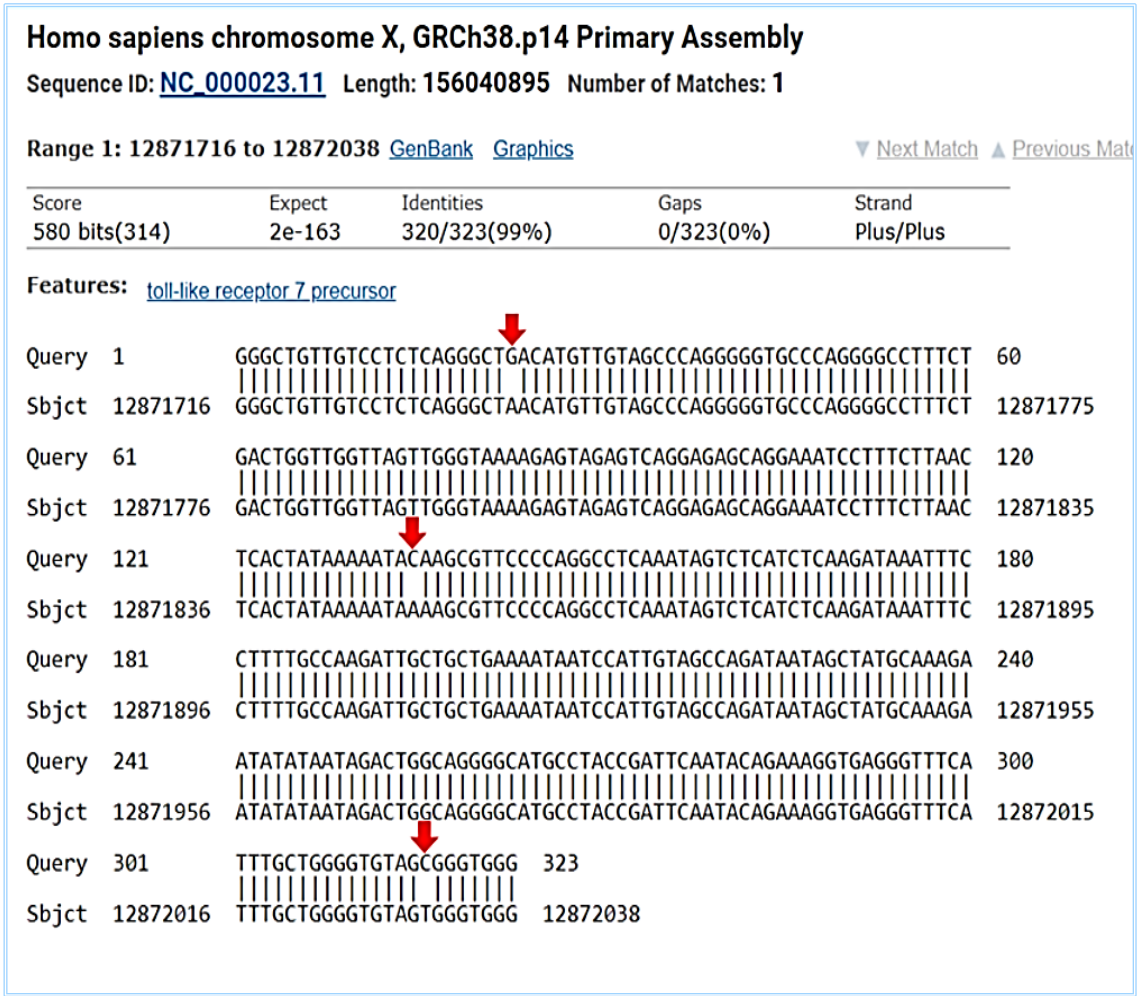
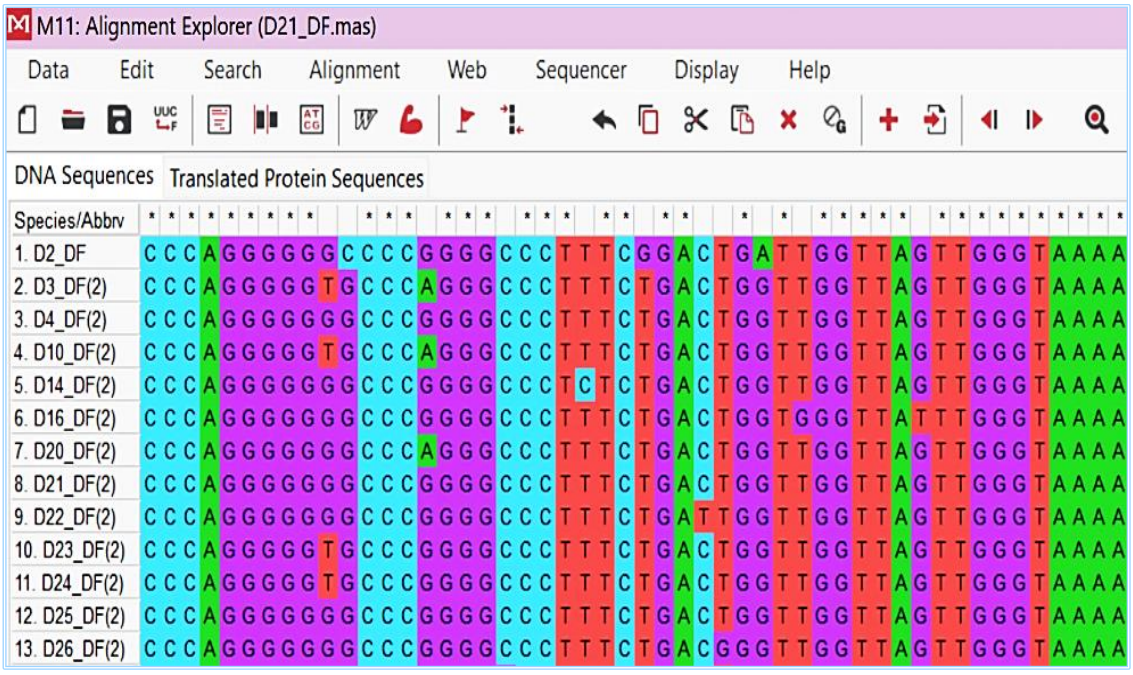


Figure (4-15): An Automated Sanger DNA Sequencing Method Showed the Electropherogram with Peaks of the Forward Strand of the Sample Sequence.

The results of the sequencing of the 20 samples were carefully examined using bioinformatics techniques, and human reference gene sequences that have previously been uploaded to the National Center for Biotechnology Information (NCBI) were aligned with them. The sequences of the 20 amplicons sent to sequencingconfirmed the specificity of primers used in this study to target regions onthe *TLR7* gene upon the results of the alignments, which were carried out by utilizing the NCBI's Basic Local Alignment Search Tool (BLAST) (Figure 4-16). Molecular Evolutionary Genetics Analysis X (MEGAX), a bioinformatics tool, was used to examine and compare the sequenced sections of the target gene. The CLUSTAL W software was used to construct multiple alignments of the sequences (Figure 4-17).



Figure(4-16): Alignment of Sequence by the BLAST Tool.



Figure(4-17): The Multiple Alignments of the Sequences were Performed by the CLUSTAL W Program.

The molecular location of variants was detected in the current study. All were registered, checked and the functional consequences of variants were predicted (Figure 4-18).

Variant ID	Chr: bp	Alleles	Class	Conseq. Type
COSV101028170	X:12888260	COSMIC_MUTATION	somatic SNV	coding sequence variant
rs179020	X:12871738	A/C/**G**/T	SNP	intron variant
rs179019	X:12871850	A/**C**/G/T	SNP	intron variant
rs985975439	X:12871888	T/**A**/C	SNP	intron variant

Figure (4-18): Molecular location and Consequences of Some Variants

By using Ensemble Genome Browser database, the locations of variantsof sequenced DNA samples all were successfully identified (Figure 4-19, Figure 4-20).

The 20 PCR products of the two regions of the *TLR7* gene were screened for variants by sequencing. The sequencing results were compared to the human reference *TLR7* gene sequence in the short arm of the X chromosome.

The (20) exon4 and (20) intron2 of DNA of study samples displayed different types of variants in the *TLR7* gene. The number of variants present in the samples was different from sample to another, the all 20 samples displayed multiple variants (Table 4-4) in their exon region sequences, while 6 out of 20 samples demonstrated double variants (Table 4-5) and the rest 14 samples displayed multiple variants (Table 4-6) in their intron region sequences.

The detected variants were divided into two groups, previously registered variants (4 variants, 1 exon4, and 3 intron2), listed in Table (4-7), and novel non-registered variants (13 variants, 8 exon4, and 5 intron2), listed in Table (4-8).

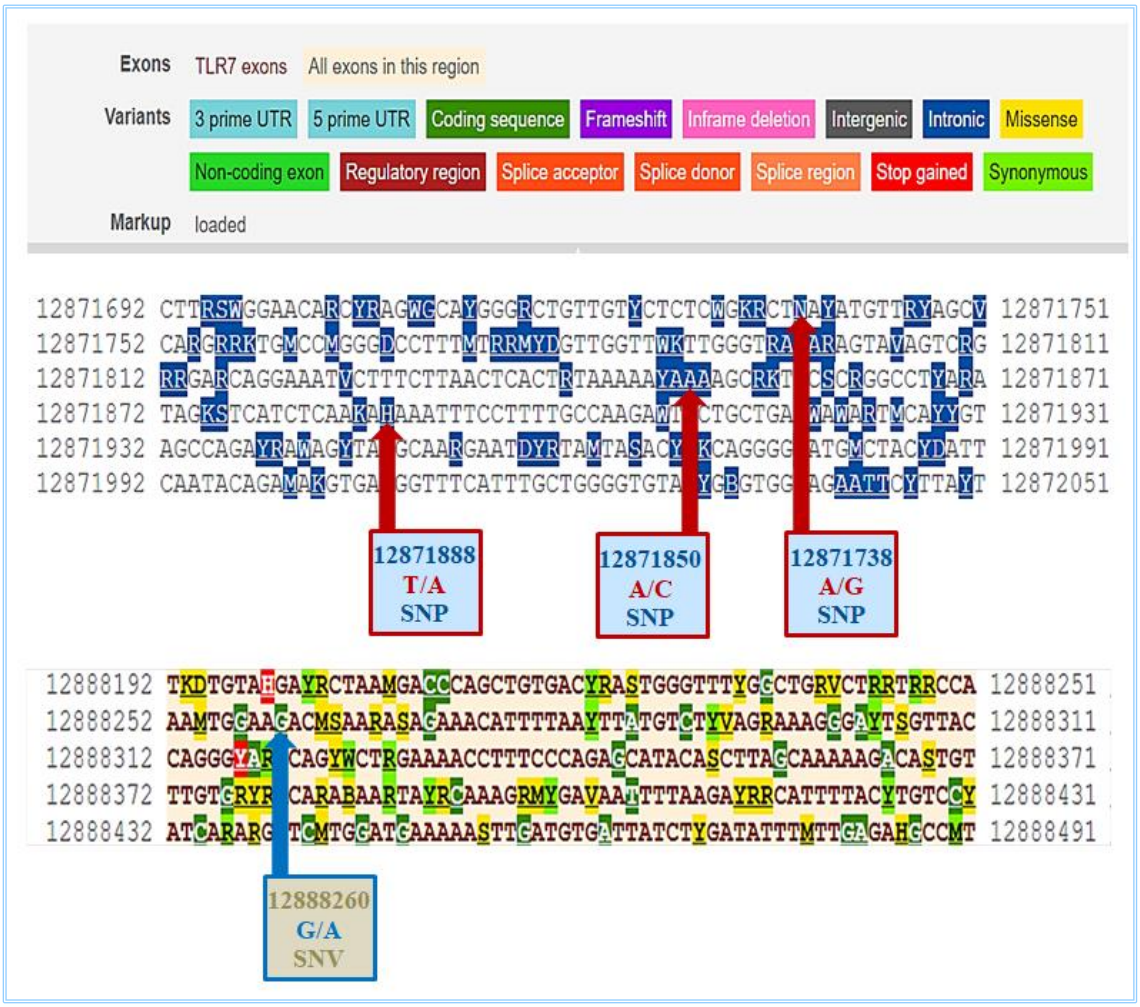


Figure (4-19): Molecular Location of Previously Registered Variants Detected in Study Samples.

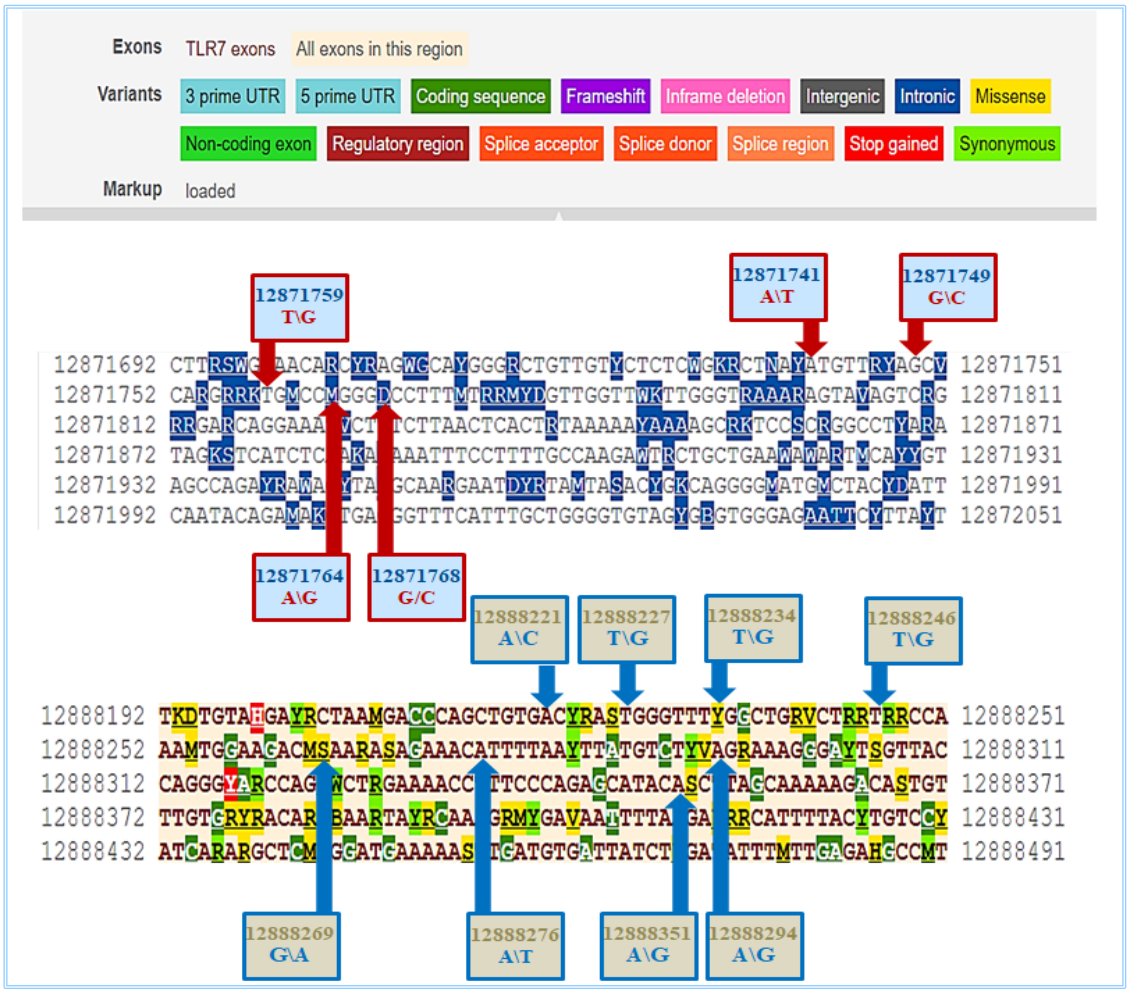


Figure (4-20): Molecular Location of New Non-Registered Variants Detected in Study Samples.

The previously registered variant [12888260 G/A] was detected in the Exon4 region, while the previously registered variants [12871738 A\G, 12871850 A\C, and 12871888 T\A] were detected in the Intron2 region (Table 4-6).

The novel non-registered variants [12888227 T\G, 12888234 T\G, 12888246 T\G, 12888294 A\G, 12888276 A\T, 12888269 G\A, 12888351 A\G, and 12888221 A\C] were detected in the Exon4 region, while the novel non-registered variants [12871741 A\T, 12871749 G\C, 12871759 T\G, 12871764 A\G, 12871768 G\C] were detected in the Intron2 region (Table 4-7).

Table(4-3): Multiple Variants on Exon4 Region Detected in Study Samples

NO.	NO. Sample	Number of Variants	Variants Location			
1	1	7	T\G	12888227	A\T	12888276
			T\G	12888234	A\G	12888351
			T\G	12888246	A\G	12888294
			G\A	12888260		
2	2	6	G\A	12888260	T\G	12888246
			T\G	12888227	A\G	12888351
			T\G	12888234	G\A	12888269
3	3	9	T\G	12888246	G\A	12888269
			A\C	12888221	A\T	12888276
			T\G	12888227	G\A	12888260
			T\G	12888234	A\G	12888294
			A\G	12888351		
4	4	6	A\C	12888221	G\A	12888260
			T\G	12888227	G\A	12888269
			T\G	12888246	A\T	12888276
5	5	8	T\G	12888227	A\G	12888294
			T\G	12888234	A\G	12888351
			T\G	12888246	G\A	12888269
			G\A	12888260	A\T	12888276
6	6	3	A\C	12888221	A\-	12888294
			T\-	12888227		
7	7	8	T\G	12888227	A\G	12888294
			T\G	12888234	A\G	12888351
			T\G	12888246	G\A	12888269
			G\A	12888260	A\T	12888276
8	8	8	A\C	12888221	A\-	12888294
			T\G	12888227	G\A	12888260
			T\G	12888234	G\A	12888269
			T\G	12888246	A\t	12888276
9	9	6	G\A	12888260	T\G	12888246
			T\G	12888227	A\G	12888351
			T\G	12888234	G\A	12888269
10	10	9	T\G	12888246	G\A	12888269
			A\C	12888221	A\T	12888276
			T\G	12888227	G\A	12888260
			T\G	12888234	A\G	12888294
			A\G	12888351		
11	11	6	A\C	12888221	G\A	12888260
			T\G	12888227	G\A	12888269
			T\G	12888246	A\T	12888276

12	12	8	A\C	12888221	G\A	12888269
			G\A	12888260	T\G	12888246
			T\G	12888227	A\T	12888276
			T\G	12888234	A\-	12888294
13	13	8	A\C	12888221	G\A	12888269
			G\A	12888260	T\G	12888246
			T\G	12888227	A\T	12888276
			T\G	12888234	A\-	12888294
14	14	6	T\G	12888227	G\A	12888260
			T\G	12888234	A\T	12888276
			T\G	12888246	A\-	12888294
15	15	6	T\G	12888227	G\A	12888260
			T\G	12888234	A\T	12888276
			T\G	12888246	A\-	12888294
16	16	7	T\G	12888227	A\T	12888276
			T\G	12888234	A\G	12888351
			T\G	12888246	A\G	12888294
			G\A	12888260		
17	17	6	G\A	12888260	T\G	12888246
			T\G	12888227	A\G	12888351
			T\G	12888234	G\A	12888269
18	18	6	G\A	12888260	T\G	12888246
			T\G	12888227	A\G	12888351
			T\G	12888234	G\A	12888269
19	19	3	A\C	12888221	A\-	12888294
			T\-	12888227		
20	20	8	A\C	12888221	A\-	12888294
			T\G	12888227	G\A	12888260
			T\G	12888234	G\A	12888269
			T\G	12888246	A\t	12888276

Table 4-4: Double Variants on Intron2 Region Appeared in Study Samples

NO.	NO. Sample	Number of Variations	Variants Location			
1	5	2	A\G	12871738	A\T	12871741
2	9		A\T	12871741	G\C	12871749
3	14		A\G	12871738	A\C	12871850
4	17		T\G	12871759	T\A	12871888
5	19		A\G	12871738	A\C	12871850
6	20		A\G	12871738	A\C	12871850

Table 4-5: Multiple Variants on intron2 Region Detected in Study Samples

NO.	NO. Sample	Number of Variants	Variants Location			
1	1	3	A\G	12871738	G\C	12871749
			A\T	12871741		
2	2	5	A\G	12871738	G\C	12871768
			A\T	12871741	A\C	12871850
			G\C	12871749		
3	3	7	A\C	12871850	T\G	12871759
			A\G	12871738	A\G	12871764
			A\T	12871741	G\C	12871768
			G\C	12871749		
4	4	3	A\G	12871738	G\C	12871749
			A\T	12871741		
5	6	5	A\G	12871738	A\G	12871764
			A\T	12871741	T\A	12871888
			T\G	12871759		
6	7	6	A\G	12871738	A\G	12871764
			A\T	12871741	G\C	12871768
			G\C	12871749	A\C	12871850
7	8	3	G\C	12871749	T\A	12871888
			T\G	12871759		
8	10	4	A\T	12871741	G\C	12871749
			G\C	12871768	T\G	12871759
9	11	5	A\G	12871738	A\G	12871764
			G\C	12871749	A\C	12871850
			T\G	12871759		
10	12	7	T\g	12871759	G\C	12871749
			A\G	12871764	A\C	12871850
			A\G	12871738	T\A	12871888
			A\T	12871741		
11	13	4	A\G	12871738	A\G	12871764
			G\C	12871749	A\C	12871850
12	15	4	A\G	12871738	T\G	12871759
			G\C	12871749	A\C	12871850
13	16	5	A\G	12871738	A\C	12871850
			T\G	12871759	T\A	12871888
			G\C	12871768		
14	18	6	G\C	12871768	A\G	12871764
			A\G	12871738	A\C	12871850
			T\G	12871759	T\A	12871888

Table 4-6 : Previously Registered Variants Detected in Study Samples

NO.	Region	Variants Location	Allele	NO. Samples	Total
1	Exon4	12888260	G\A	1,2,3,4,5,7,8,9,10,11,12,13,14,15,16,17,18,20	18
2	Intron2	12871738	A\G	1,2,3,5,6,7,11,12,13,14,15,16,18,19,20	15
3	Intron2	12871850	A\C	2,3,7,11,12,13,14,15,16,18,19,20	12
4	Intron2	12871888	T\A	6,8,12,16,17,18	6

Table 4-7 : New Non-Registered variants Detected in Study Samples

NO.	Region	Variants Location	Allele	NO. Samples	Total
1	Exon4	12888227	T\G	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18, 19,20	20
2	Exon4	12888246	T\G	1,2,3,4,5,7,8,9,10,11,12,13,14,15,16,17,18,20	18
3	Exon4	12888234	T\G	1,2,3,5,7,8,9,10,12,13,14,15,16,17,18,20	16
4	Exon4	12888294	A\G	1,3,5,6,7,8,10,12,13,14,15,16,19,20	14
5	Exon4	12888276	A\T	1,3,4,5,7,8,10,11,12,13,14,15,16,20	14
6	Exon4	12888269	G\A	2,3,4,5,7,8,9,10,11,12,13,17,18,20	14
7	Exon4	12888351	A\G	2,4,5,7,9,10,16,17,18	10
8	Exon4	12888221	A\C	3,4,6,8,10,11,12,13,19,20	10
9	Intron2	12871759	T\G	3,6,8,10,11,12,15,16,17,18	10
10	Intron2	12871749	G\C	1,2,3,7,8,9,10,11,12,13,15	11
11	Intron2	12871741	A\T	1,2,3,5,6,7,9,10,12	9
12	Intron2	12871764	A\G	3,6,7,11,12,13,18	7
13	Intron2	12871768	G\C	2,3,7,10,16,18	6

4.4.2. Effect of *TLR7* Gene variants on Study Parameter

The effects of *TLR7* gene variants on study parameters (CEA, CA125, CA15-3, and CA27-29) were investigated by comparing the levels of each tumor marker in the samples sharing the same variants. A point biserial correlation coefficient was conducted to examine the relationship between each particular type of SNPs (whether in the exons or introns) and the four tumor markers (CEA, CA125, CA15-3, and CA27-29).

It was found that the substitution G/C (12871749) of the intron2 region was moderately negatively and statistically significantly correlated with the CEA tumor marker serum levels (point biserial correlation coefficient = -0.396, p = 0.03).

Furthermore, there was a statistically significant correlation (p = 0.015) between the SNP A/C (12888221) of the exon4 region and the CA125 tumor marker serum value, the interaction was of an intermediate correlation (point biserial correlation coefficient = -0.442).

The substitution A/G (12871764) of the intron2 region was moderately associated with the CA15-3 tumor marker levels (point biserial correlation coefficient = -0.385), and the two variables were statistically significantly associated (p = 0.036). The results revealed that there were no other significant, weak, intermediate, or strong associations among all other types of substitutions when tested individually for the four tumor markers under study.

The effects of *TLR7* gene variants on this study's biochemical markers (CEA, CA125, CA15-3, and CA27-29) were investigated. This was done by comparing the levels of each marker, grouped by the number of variants. Related to the exon4 region results, the samples were divided into two groups based on the number of variants in each sample. The first group includes samples that contain fewer than 6 variants, and the second group comprises samples that contain more than 6 variants, with each group containing 10 samples. Related to the intron2 region results, the results of the samples were divided into three groups according to the number of variants in each sample. The first group includes the samples that contain fewer than 3 variants; the second group consists of the samples that contain 3–5 variants; and the third group includes the samples that contain more than 5 variants.

The results of the present study reveal (50%) less than 6 variants and (50%) more than 6 variants for Exon4 region (Tables 4-9) , while the results of Intron2 region (45%) less than 3 variants, (35%) variants between 3-5 and (20%) variants more than 5 (Tables 4-10).

Table 4-8 : Distribution of Sample Study According to Genetic Results (Exon4)

	No. of variants	No. of samples	Percentage (%)
Exon	<6	10	50%
	>6	10	50%
	Total	20	100%

Table 4-9 : Distribution of Sample Study According to Genetic Results (Intron2)

	No. of variants	No. of samples	Percentage (%)
Intron	<3	9	45%
	3-5	7	35%
	>5	4	20%
	Total	**20**	**100%**

The table (4-9) contains two sets of data that were expressed in different manners, the first set of data Included the (CEA) and (CA 27-29) biomarkers, they were represented median ± Interquartile range (IQR) because they were not normally distributed and this also implied the use of independent T test alternative (Mann-whitney U test) to compare between the two groups (<6 and >6). While the second set of data Included (CA125) and (CA15-3) were normally distributed; hence, they were represented by Mean ± standard error of the mean (SE) and were compared by independent samples T-test.

Related to the exon4 results, the result displayed significant differences between the same groups in the level of CEA (p value= 0.015), while the result displayed non-significant differences between the two groups (<6 and >6) in the level of CA 125, CA 15-3, and CA 27-29 with a p value (0.44), (0.96), and (0.48) respectively (Table 4-11).

Table (4-10): The Association between the Number of Variants of Exon4 *TLR7* Gene with Study Biochemical markers

No. variants	CEA (Median±IQR)	CA125 (Mean±SE)	CA 15-3 (Mean±SE)	CA 27-29 (Median±IQR)
<6	32.44±28.12	19.73±4.97	115.64±36.52	283.58±173.23
>6	52.42±38.52	15.29±2.81	117.74±30.53	255.1±43.11
P-value	0.015*	0.44	0.96	0.48
*(P≤0.05) Significant by Mann-Whitney U test.				

Related to the intron2 region (Table 4-12), the result showed the non-significant differences between the three groups (<3, 3-5, and >5) in the level of CEA, CA 125, CA 15-3, and CA 27-29 with a p value(0.56), (0.89), 0.14) and (0.69) respectively.

Table(4-11): The Association between the Number of Variants of Intron2 *TLR7* Gene with Study Biochemical markers

No. Variants	CEA (Median±IQR)	CA125 (Median±IQR)	CA15-3 (Median±IQR)	CA27-29 (Mean±SE)
<3	271.26±162.4	11.2±14.8	65.3±24.4	45.59±6.67
3-5	276.2±97.2	11.1±2.6	112.5±218.7	38.44±8.85
>5	339.7±170.0	21.3±24.0	93.5±187.2	49.0±9.35
P-value	0.56	0.89	0.14	0.69
by Kruskal-Wallis test and one-way ANOVA				

In this study, regarding the results of the exon4 region, the samples were divided into two groups based on the status of variants present (either previously registered variants or new, non-registered variations). The first group (New) included samples that contained new, non-registered (novel) variants only, while the second group (Mixed) included samples that contained previously registered variants and new, non-registered variants. The results showed non-significant differences between the new and mixed variants in the level of CEA, CA 125 and CA 27-29, with a p-value 0.76, 0.32 and 0.428, respectively. However, the CA15-3 result showed significant differences between new and mixed variants, with a p-value of 0.02 (Table 4-13), (Figure 4-22).

Table(4-12): Mean Values Differences of Biochemical Markers According to Different Variants (Exon4),(n=20)

Variants status	N.	CEA (Median±IQR)	CA125 (Median±IQR)	CA15-3 (Median±IQR)	CA27-29 (Mean±SE)
New	2	274.0±0	31.9±0	52.4±0	32.99±10.2
Mixed	18	269.9±164.9	11.24±8.25	81.47±49.4	44.9±5.0
P-value		0.76	0.32	0.02*	0.428

*(P≤0.05) Significant by Mann-Whitney U test.

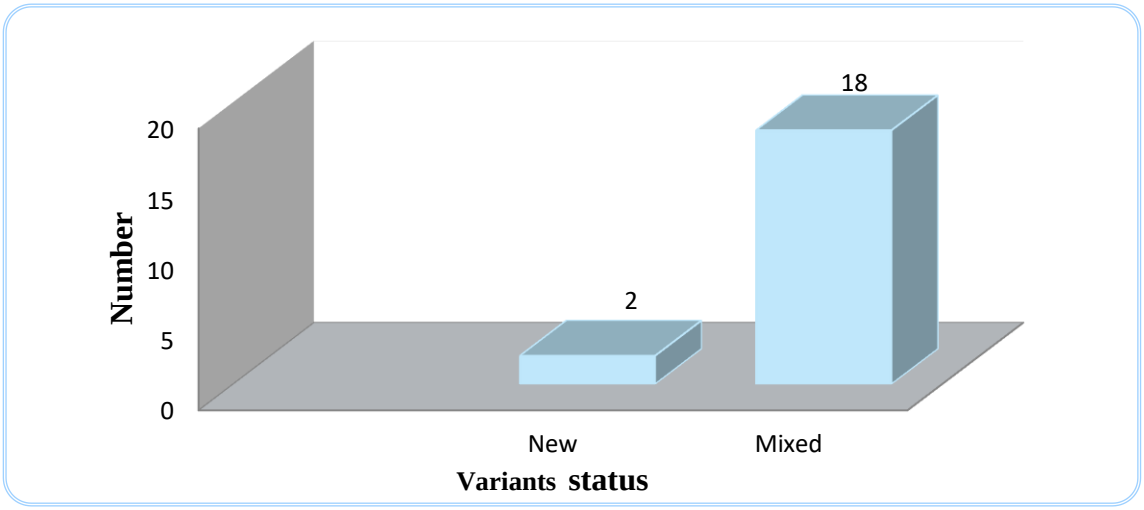


Figure (4-21): Distribution of Sample Study Depending on Variants (Exon4), (n=20).

On the other hand, regarding the results of the intron2 region, the samples were divided into three groups based on the status of variants present (either previously registered variants or new, non-registered variations). The first group (New) included samples that contained new, non-registered (novel) variants only, the second group (Mixed) included samples that contained previously registered variants and new, non-registered variations, while the third group (Mixed) included samples that contained previously registered variants only. The result showed non-significant differences between the new, mixed, and old variants in the level of CEA, CA125, CA15-3, and CA27-29, with a p-value 0.64, 0.87,

0.35, and 0.92, respectively (Table 4-14; Figure 4-23).

Table(4-13): Mean Values Differences of Biochemical Markers According to Different variants (Intron),(n=20).

variants status	N.	CEA (Mean±SE)	CA 125 (Median±IQR)	CA 15-3 (Median±IQR)	CA 27-29 (Mean±SE)
New	2	271.0±17.81	11.12±0	81.94±0	43.29±18.19
Mixed	15	293.95±27.81	11.62±20.5	81.77±52.5	44.76±5.9
Old	3	356.5±96.45	11.15±0	58.82±0	39.15±4.61
P-value		0.64	0.87	0.35	0.92
by Kruskal-Wallis test and one way ANOVA					

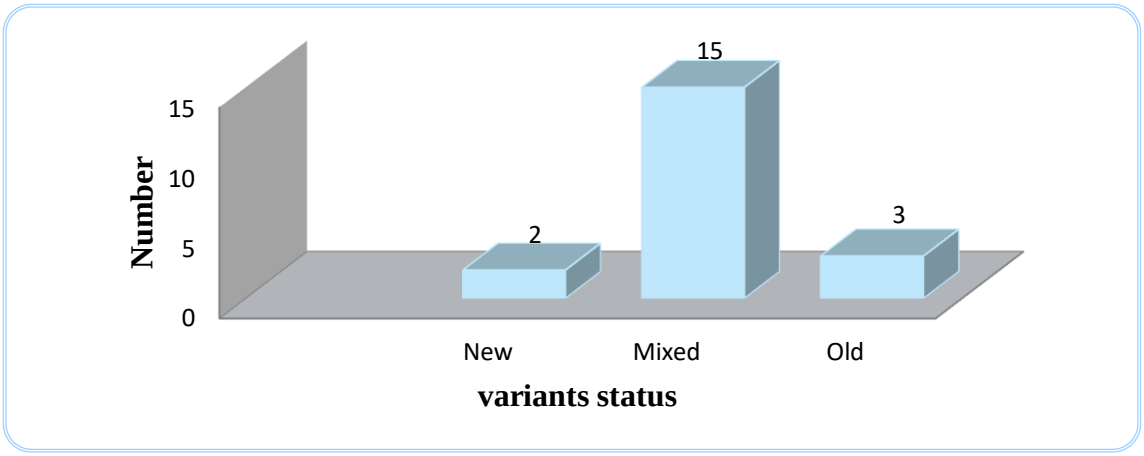


Figure (4-22): Distribution of Sample Study Depending on variants (Intron),(n=20).

The significance of variants in *TLR7* genes for oncogenomics remains unclear. There was a dearth of studies analyzing the association of *TLR* gene variants with cancer, despite the abundance of studies devoted to the association between variants of genes encoding proteins of the *TLR* pathway with various diseases (Kutikhin A. G. 2011). The most researched pattern recognition receptors were toll-like receptors (*TLRs*). increasing data suggests a significant correlation between *TLRs* and the incidence and progression of breast cancer. Accordingly, focusing on these receptors might be a useful therapy approach for breast cancer (Shi *et al.* 2020).

(Kidd *et al*., 2013), According to their findings, *TLRs* were extensively expressed in breast cancer cells, and these receptors may be activated to cause aggressive tumor activity, as well as cell invasion, proliferation, migration, and metastasis.

This is inconsistent with those reported by (Shi et al., 2020), who report that, compared with the normal control tissues, *TLR6*, *TLR7*, and *TLR8* expression levels were marginally elevated in breast cancer tissues.

Several studies have indicated that *TLR7* activation was involved in the pathogenesis of cancer, where *TLR7* can promote the proliferation of the cancer cells (Ochi et al. 2012). However, other studies, including the study of (Chi *et al*., 2017), revealed that *TLR7* can induce apoptosis in some special cancer cells that play the role of antitumors, where the *TLR7* agonists were small molecules that stimulated innate immune cells, leading to the activation of humeral and cellular immunity, thus engendering a series of anti-tumor activities.

Few previous studies were done about the relationship and effect of *TLR7* gene variants on breast cancer. Regarding rs179019, which was one of the present study-reviewed variants, there were no previous studies revealing association between the variant and breast cancer, but it was linked to other diseases, such as the study conducted by (Kutikhin *et al.*, 2011) that showed association with SLE in Japanese women. This finding corresponds with (Ranjan *et al.*, 2023) results that showed a significant association between the rs179019 variant and susceptibility to SLE development. This association might be explained by overproduction of pro-inflammatory cytokines in addition to type I interferon. (Zhang *et al*., 2019) showed no association between the rs179019 variant and Enterovirus 7I (EV7I) Infection, Hand, Foot, and Mouth Disease (HFMD).

The second *TLR7* gene variant, rs179020, which was detected in this study results and registered previously in another study with a link with diseases rather than breast cancer, (Traks *et al.*, 2015), in a study on vitiligo disease (a chronic disease characterized by lightening of the skin), showed

an association between the rs179020 variant and this disease. While (Galimova *et al*., 2017), in a study on psoriasis disease, revealed an association between the risk of developing this disease and the rs179020 variant. In accordance with this finding, (Al-Humairi et al., 2019) showed an association between this variant and a potential risk for urinary bladder cancer (UBC). As for the third and fourth *TLR7* gene variants (rs985975439, COSV101028170), which were detected in this study results, there were no previous studies revealing association between these variants and breast cancer or another disease.

Finally, we thought that the results of this study, and particularly the thirteen novel variants that were found in the *TLR7* gene's exon4 regions (12888227 T\G, 12888234 T\G, 12888246 T\G, 12888294 A\G, 12888276 A\T, 12888269 G\A, 12888351 A\G, 12888221 A\C) and intron2 regions (12871741 A\T, 12871749 G\C, 12871759 T\G, 12871764 A\G, 12871768 G\C), might gain importance in the field of *TLR7* gene studies due to the presence of these variants in the important coding and non-coding regions.

Furthermore, the three variants, 12871749 G\C,12871764 A\G, and 12888221 A\C, among the thirteen novel variants, might be the most important variants because this variant showed a significant effect on some study parameters. To identify precisely their role in breast cancer patients, more studies need to be further studied in future research. It must be noted that it has to point out that the study results were limited by the relatively small sample size of patients and controls, suggesting the need for large-scale studies to corroborate the results and warrant the validation of findings.

Conclusions and Recommendations

Conclusions

Based on the results of the present study, the following points can be concluded:

1. A significant effect of *TLR7* - 12871749 G\C variant of the Intron region on serum level of CEA.
2. A significant effect of *TLR7* - 12871764 A\G variant of the Intron region on serum level of CA 15-3.
3. A significant effect of *TLR7* - 12888221 A\C variant of the Exon region on serum level of CA 125.
4. The three variants, 12871749 ,12871764 and 12888221 might be the most important variants among the thirteen novel variants.

Recommendations

In the light of results and conclusions, the following points can be recommended:

1. Further work with a larger population was needed to validate the *TLR7* gene effect on Breast cancer.
2. Further intensive research on the 12871749 G\C ,12871764 A\G and 12888221 A\C variants.
3. Study the extent of the effect of the *TLR7* gene and its variants in Breast cancer patients and the extent on early diagnosis .
4. The study suggest continuing to find out if there were variants in the rest of the gene regions in Breast cancer patients Iraqi population and their Explain its effect.
5. The study suggest execution more researches on role of *TLR7* and *TLR7* pathway SNPs in the fields of tumor immunology, may open new avenues in cancer biology and cancer prevention.
6. The study suggests execution of more future well-designed studies should shed light on the significance of the variants of genes encoding *TLRs* and proteins of the *TLRs* pathway especially *TLR7* for programs of cancer prevention based on genomic risk markers also, to clarify what role *TLR7* agonists could play as antitumor treatments.

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The Appendices

Appendix 1 : Questionnaire form for Breast cancer

"Questionnaire form for Breast cancer"

Zahraa Majid

Patient No.									
The date	/ /								
Patient Name									
The address									
Age at enrollment in the study					Age at diagnosis				
Weight	Kg.			Height		cm.			
Marital history	single		Married						
drug history	oral contraceptive pills						Yes	No	
obstetrical history	number of children			lactation			Yes	No	
Stage of breast cancer	Stage 1		Stage 2		Stage 3		Stage 4		
indicate any Breast Symptoms you are currently experiencing	Mass or Lump :		Nipple Discharge :		Breast Pain :		Other :		
	Yes	No	Yes	No	Yes	No	Yes	No	
Side	Left				Right				
Breast hormonal receptor	PR.				ER.				
Her2									
Lumpectomy							Yes	No	
Mastectomy							Yes	No	
Have you ever had a previous breast biopsy ?							Yes	No	
Did you receive chemotherapy?							Yes	No	
Do you have a history of prior breast cancer ?							Yes	No	
							if Yes , Side:	Left	Right
Do you have a family history of breast cancer?							Yes	No	
Do you have a family history of other cancers?							Yes	No	
Smoking							Yes	No	

Body mass index	Under weight	normal weight	Overweight (pre-obese)	obesity (first degree)	Obesity (second degree)	obesity (third degree)
	17.0 – 18.4	18.5 – 24.9	25.0 – 29.9	30.0 – 34.9	35.0 – 39.9	≥ 40.0

Appendix 2 : The PCR Programs Optimization

Program 1

Reagent	Volume	
Primer F	1	µl
Primer R	1	µl
DNA	3	µl
D.W.	13.5	µl
Master Mix	6.5	µl

Steps	Stage	cycle	Step	temp.	Time
Initial denaturation	1	1	1	94.0	4:00
Denaturation	2	35	1	94.0	0:30
Annealing			2	60.0	0:30
Extension			3	72.0	0:30
Final extension	3	1	1	72.0	5:00
Hold			2	4.0	HOLD

Program 2

Reagent	Volume	
Primer F	1	µl
Primer R	1	µl
DNA	3	µl
D.W.	13.5	µl
Master Mix	6.5	µl

Steps	Stage	cycle	Step	temp.	Time
Initial denaturation	1	1	1	94.0	4:00
Denaturation	2	35	1	94.0	0:30
Annealing			2	59.0	0:30
Extension			3	72.0	0:30
Final extension	3	1	1	72.0	5:00
Hold			2	4.0	HOLD

Program 3

Reagent	Volume	
Primer F	2	µl
Primer R	2	µl
DNA	5	µl
D.W.	8	µl
Master Mix	8	µl

Steps	Stage	cycle	Step	temp.	Time
Initial denaturation	1	1	1	94.0	4:00
Denaturation	2	35	1	94.0	0:30
Annealing			2	59.5	0:30
Extension			3	72.0	0:30
Final extension	3	1	1	72.0	5:00
Hold			2	4.0	HOLD

Program 4

Reagent	Volume	
Primer F	2	µl
Primer R	2	µl
DNA	5	µl
D.W.	8	µl
Master Mix	8	µl

Steps	Stage	cycle	Step	temp.	Time
Initial denaturation	1	1	1	94.0	4:00
Denaturation	2	35	1	94.0	0:30
Annealing			2	59.5	0:45
Extension			3	72.0	0:30
Final extension	3	1	1	72.0	5:00
Hold			2	4.0	HOLD

Program 5

Reagent	Volume	
Primer F	2	µl
Primer R	2	µl
DNA	5	µl
D.W.	8	µl
Master Mix	8	µl

Steps	Stage	cycle	Step	temp.	Time
Initial denaturation	1	1	1	94.0	4:00
Denaturation	2	35	1	94.0	0:30
Annealing			2	59.5	0:30
Extension			3	72.0	0:45
Final extension	3	1	1	72.0	5:00
Hold			2	4.0	HOLD

Program 6

Reagent	Volume	
Primer F	2	µl
Primer R	2	µl
DNA	5	µl
D.W.	8	µl
Master Mix	8	µl

Steps	Stage	cycle	Step	temp.	Time
Initial denaturation	1	1	1	94.0	4:00
Denaturation	2	35	1	94.0	0:30
Annealing			2	58.5	0:45
Extension			3	72.0	0:45
Final extension	3	1	1	72.0	5:00
Hold			2	4.0	HOLD

Program 7

Reagent	Volume	
Primer F	2	µl
Primer R	2	µl
DNA	5	µl
D.W.	8	µl
Master Mix	8	µl

Steps	Stage	cycle	Step	temp.	Time
Initial denaturation	1	1	1	94.0	4:00
Denaturation	2	35	1	94.0	0:30
Annealing			2	58.0	0:45
Extension			3	72.0	0:30
Final extension	3	1	1	72.0	5:00
Hold			2	4.0	HOLD

Program 8

Reagent	Volume	
Primer F	2	µl
Primer R	2	µl
DNA	6	µl
D.W.	7	µl
Master Mix	8	µl

Steps	Stage	cycle	Step	temp.	Time
Initial denaturation	1	1	1	94.0	4:00
Denaturation	2	35	1	94.0	0:30
Annealing			2	57.0	0:45
Extension			3	72.0	0:30
Final extension	3	1	1	72.0	5:00
Hold			2	4.0	HOLD

Program 9

Reagent	Volume	
Primer F	2	µl
Primer R	2	µl
DNA	6	µl
D.W.	8	µl
Master Mix	7	µl

Steps	Stage	cycle	Step	temp.	Time
Initial denaturation	1	1	1	94.0	4:00
Denaturation	2	40	1	94.0	0:30
Annealing			2	57.0	0:45
Extension			3	72.0	0:30
Final extension	3	1	1	72.0	5:00
Hold			2	4.0	HOLD

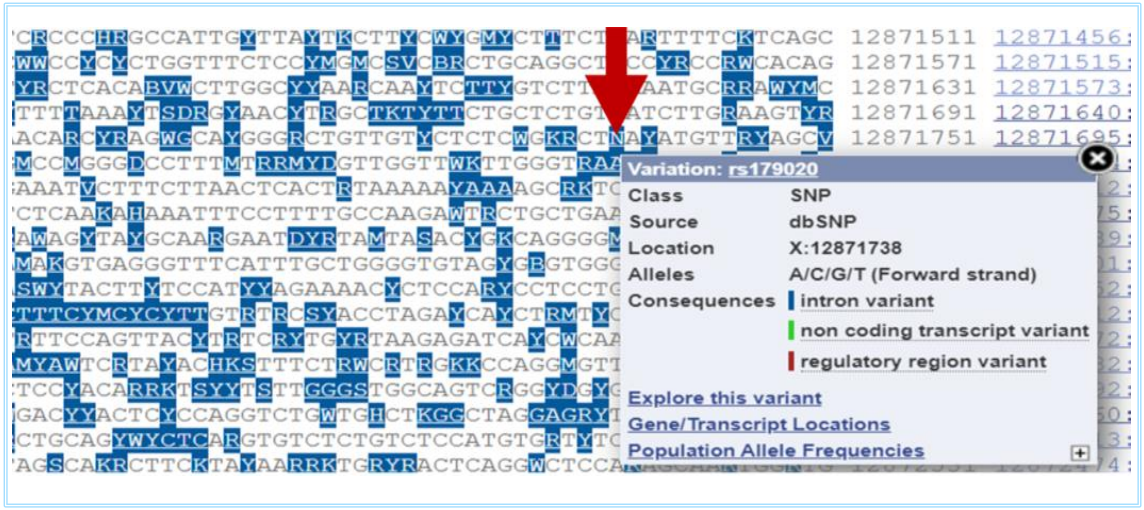
Program 10

Reagent	Volume	
Primer F	2	µl
Primer R	2	µl
DNA	8	µl
D.W.	6	µl
Master Mix	7	µl

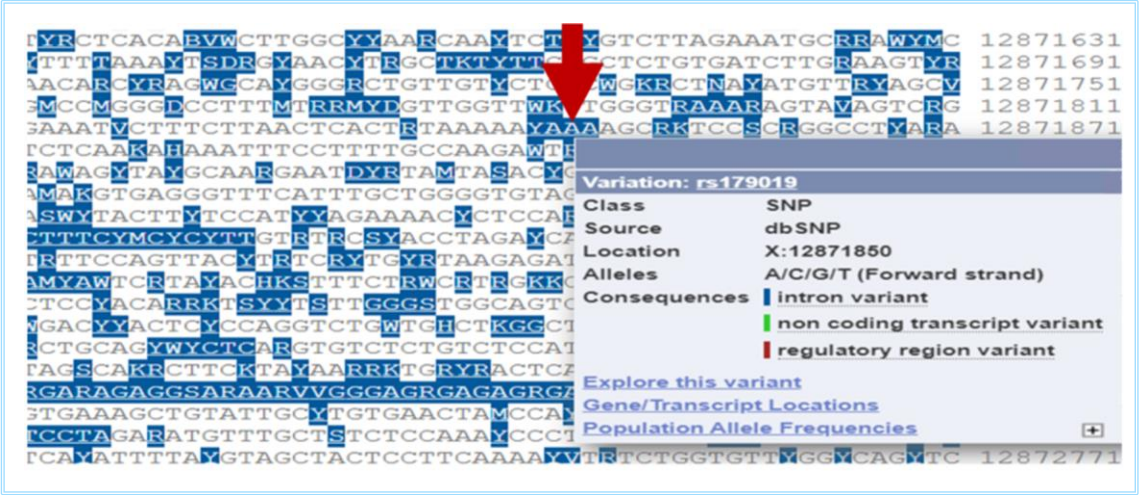
Steps	Stage	cycle	Step	temp.	Time
Initial denaturation	1	1	1	94.0	4:00
Denaturation	2	35	1	94.0	0:30
Annealing			2	57.0	0:45
Extension			3	72.0	0:30
Final extension	3	1	1	72.0	5:00
Hold			2	4.0	HOLD

Appendix 3 : Detect Previously Registered Variant in Study Sample (12888260 G\A)

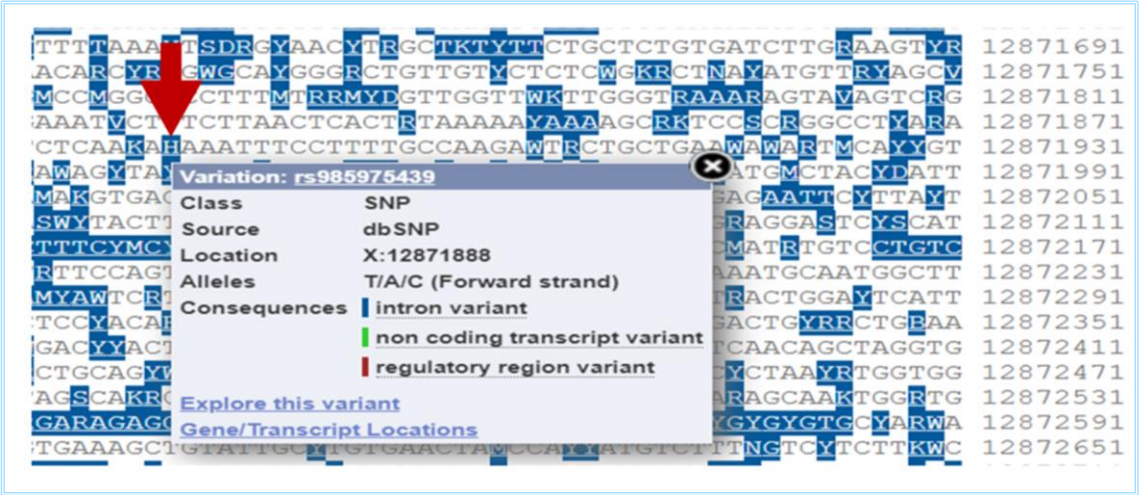
Appendix 4 : Detect Previously Registered Variant in Study Sample (12871738 A\G)



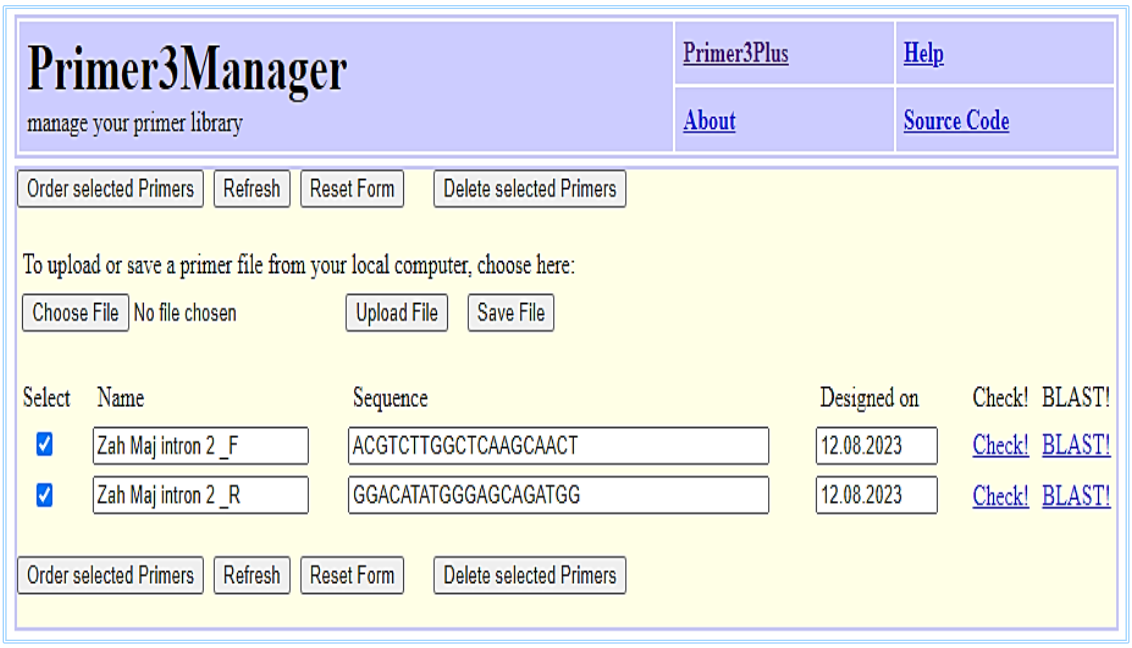
Appendix 5 : Detect Previously Registered Variant in Study Sample (12871850 A\C)



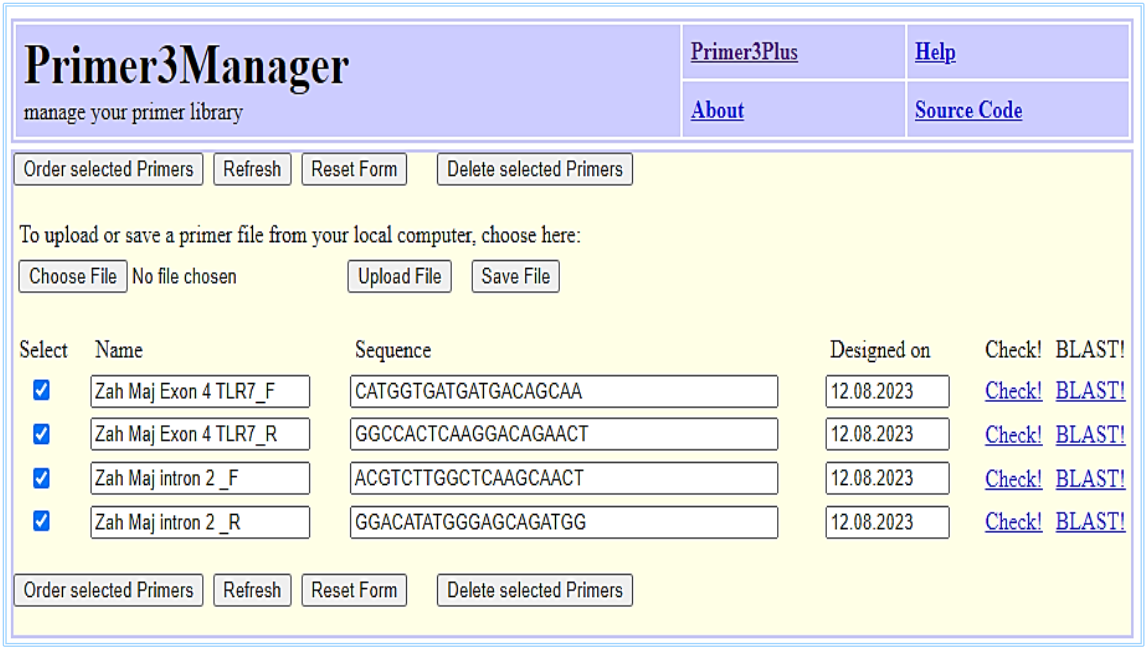
Appendix 6 : Detect Previously Registered Variant in Study Sample (12871888 T\A)



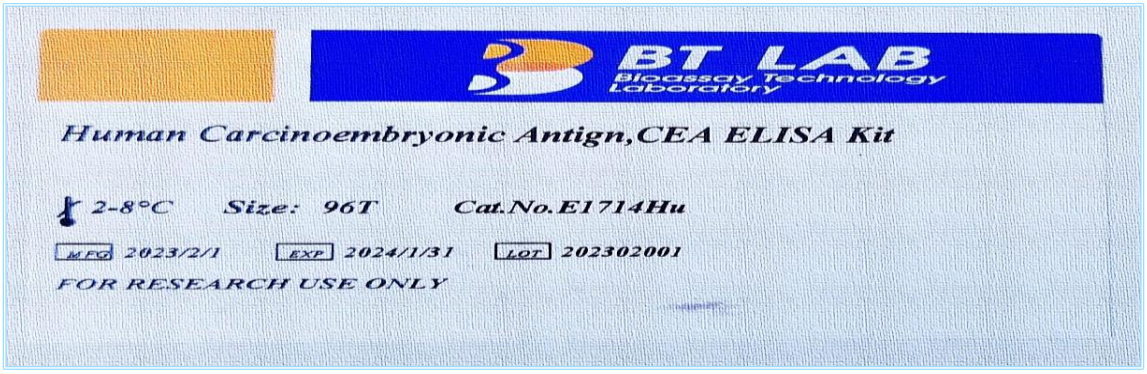
Appendix 7 : Primer Design Intron2 *TLR7* Gene Primer Manager Program.



Appendix 8 : Primer Design Exon 4 *TLR7* Gene Primer Manager Program.



Appendix 9: Biochemical Markers kits.



الخلاصة

أجريت الدراسة الحالية في قسم المختبرات السريرية / كلية العلوم الطبية التطبيقية / جامعة كربلاء خلال الفترة من تشرين الثاني 2022 الى نيسان 2024. هدفت الدراسة إلى إلقاء الضوء على العلاقة بين تباين جين *TLR7* ومستوى بعض المؤشرات الكيموحيوية (CA15-3 و CEA و CA125 و CA27-29) لدى مريضات سرطان الثدي من النساء العراقيات. تم اخذ 100 امرأة متطوعة ، في هذه الدراسة ، 50 امرأة مصابة بسرطان الثدي ، في المقابل تم اخذ 50 امرأة ظاهراً تتمتع بصحة جيدة ، تتراوح أعمارهن بين 29 إلى 75 عاماً في وقت التحقيق.

تم جمع عينات الدم من المتطوعات في مركز الامام الحسين (ع) لعلاج الأورام وأمراض الدم في مدينة كربلاء المقدسة / العراق ، وتم التوقيع على الموافقة الأخلاقية من قبل كل متطوع.

كانت الدراسة عبارة عن دراسة وراثية سكانية، أجريت على مجموعتين من العينات. تم التحقيق في تباين جين *TLR7* بواسطة طريقة التسلسل Sanger. تم قياس مستويات (CA15-3 و CEA و CA125 و CA27-29) في المصل بواسطة تقنية (ELISA).

أظهر التحليل الإحصائي فروقًا مهمة إحصائياً (فروق معنوية) عند مقارنة مستويات علامات الورم الأربعة وهي (CEA و CA125 و CA 15-3 و CA 27-29) في المصل بين مجموعة السيطرة ومجموعة المرضى ($P<0.001$).

أظهر التحليل الجيني لنتائج 20 عينة (تم اختيارها من عينات مرضى سرطان الثدي) اختلافات متنوعه في شكل جين *TLR7* ، وكان عدد المتغيرات الموجودة في العينات مختلفًا من عينة إلى أخرى، حيث اظهرت جميع العينات اختلافات متعددة في التسلسل الجيني لمنطقة Exon4 ، بينما اظهرت 6 من أصل 20 عينة اختلافات مزدوجة في تسلسلها الجيني و اظهرت العينات الـ 14 المتبقية اختلافات متعددة في التسلسل الجيني لمنطقة Intron2.

تم تقسيم المتغيرات المكتشفة إلى مجموعتين، متغيرات مسجلة مسبقًا (4 متغيرات (1 لمنطقة Exon4 و3 لمنطقة Intron2)) ، ومتغيرات جديدة غير مسجلة (13 متغيرًا (8 لمنطقة Exon4 و 5 لمنطقة Intron2)).

تم اكتشاف المتغيرات المسجلة مسبقًا (G/A 12888260) في 10 عينات تم اكتشافها في منطقة Exon4 ، بينما في منطقة Intron2 تم اكتشاف المتغيرات (A\G 12871738 ، A\C 12871850 ، T\A 12871888) في 18 عينة.

تم اكتشاف المتغيرات الجديدة غير المسجلة (T/G 12888260 ، T/G 12888246 ، A/G 12888294 ، A/T 12888276 ، T/G 12888234 ، G/A 12888269 ، 12888351 A/G ، A/C 12888211) في 12 عينة تم اكتشافها في منطقة Exon4 ، بينما في منطقة Intron2 تم اكتشاف المتغيرات (T/G 12871759 ، G/C 12871794 ، A/T 12871741 ، 12871768 G/C ، A/G 12871764) في 17 عينة.

تم التحقيق في تأثيرات الاختلافات الجينية *TLR7* على عوامل الدراسة (CA15-3 و CEA و CA125 و CA27-29) من خلال مقارنة مستوى أو كمية كل عامل في العينات التي تشترك في نفس الاختلاف. من خلال تحليل النتائج أظهرت أن المتغير(G/C 12871749) لمنطقة Intron2 كان مرتبطًا إلى حَدٍّ ما بشكل سلبي إحصائيًا مع مستويات CEA (معامل الارتباط الثنائي النقطة = -0.396، p = 0.03). علاوة على ذلك، يعرض التحليل الإحصائي وجود فروقاً ذات دلالة إحصائية (فروق معنوية) (p = 0.015) بين المتغير (A/C 12888211) لمنطقة Exon4 وقيمة CA125 ، وكان التفاعل ذو ارتباط متوسط (معامل الارتباط الثنائي النقطي=- 0.442). أظهرت النتائج ارتباط المتغير (A/G 12871764) لمنطقة Intron2 بشكل معتدل بمستويات علامة الورم CA15-3 (معامل الارتباط الثنائي النقطة = -0.385) وكان المتغيران مرتبطين بشكل كبير إحصائيًا (p = 0.036).

أظهرت نتائج منطقة Exon4 فروقاً ذات دلالة احصائية (فروق معنوية) بين نفس المجموعتين في مستوى CEA قيمة (p = 0.015)، بينما أظهرت النتائج فروقاً غير معنوية بين المجموعتين مقسمة حسب عدد الطفرات (<6 و>6) في مستوى CA125، CA15-3 وCA27-29 بقيمة p (0.44)، (0.96) و (0.48) على التوالي. أظهر التحليل الإحصائي وجود فروقاً غير مهمة (فروق غير معنوية) بين المتغيرات الجديدة والمختلطة في مستوى (CEA وCA125 وCA27-29) بقيمة p (0.76) و(0.32) و(0.428) على التوالي. في حين أظهرت نتيجة CA 15-3 فروقاً ذات دلالة احصائية (فروق معنوية) بين المتغيرات الجديدة والمختلطة حيث كانت قيمة(p=0.02) في منطقة Exon4.

جامعة كربلاء

كلية العلوم الطبية التطبيقية

قسم التحليلات المرضية

علاقة تغايرات جين TLR7 مع بعض المؤشرات الكيموحيوية بين النساء المصابات بسرطان الثدي

رسالة مقدمة

الى مجلس كلية العلوم الطبية التطبيقية – جامعة كربلاء

وهي جزء من متطلبات نيل شهادة الماجستير في التحليلات المرضية

كُتبت بواسطة

زهراء ماجد جاسب

بكالوريوس تحليلات مرضية / كلية الصفوة الجامعة ، 2018

بأشراف

أ.د. جودت نوري غائب

صفر (1446) **اب (2024)**