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University of Babylon - College of Science

Department of Biology

# Genetic Polymorphism of Some Cytokines Associated With Female Breast Tumor

A Thesis

Submitted to the Council of College of Science-University of Babylon as a Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy in biology

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**جمهـــــــــورية العـــــــــراق**
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# تعدد الأشكال الوراثية لبعض السيتوكينات المرتبطة بأورام الثدي عند النساء

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*Wurud Ali 2023*

# *Dedication*

*TO My mother*

*Wurud Ali 2023*

بِسمِ اللهِ الرَّحمَنِ الرَّحِيمِ

﴿وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ
رَبِّيْ وَمَا أُوْتِيْتُمْ مِنَ الْعِلْمِ إِلاَّ قَلِيْلاً﴾

صَدقَ اللهُ العَليُّ العَظِيم

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## List of Abbreviations

| Abbreviated Form | Meaning |
|---|---|
| BRCA | BReast Cancer gene 1 |
| BMI | Body max index |
| IGF-I | Insulin-like growth factor I |
| UDH | usual ductal hyperplasia |
| FEA | flat epithelial atypia |
| CCL | columnar cell lesions |
| CDCA7 | Cell Division Cycle Associated 7 |
| p53 | Tumor protein |
| Bcl-2 | B-cell lymphoma 2 Bcl-2-associated athanogene-1 |
| BAG-1 | Bcl2 associated athanogene 1 |
| Bax | BCL2 Associated X Finite Element Analysis |
| FEA | Finite element analysis |
| DCIS | Ductal carcinoma in situ |
| IDC | Invasive ductal carcinoma |
| DCIS | Ductal carcinoma in situ |
| IDC-NST | Invasive ductal carcinoma no specific type |
| ILC | invasive lobular carcinoma |
| BC | Breast cancer |
| ECM | extracellular matrix tumor microenvironment |

| | |
|---|---|
| **TME** | **Tumor microenvironment** |
| **Ags** | **Antigens** |
| **MHC** | **Major histocompatibility** |
| **APCs** | **Antigen presenting cell** |
| **TCRs** | **T cell receptors** |
| **OS** | **overall survival** |
| **PFS** | **progression-free survival** |
| **PCR** | **Polymerase chain reaction** |
| **HER2+** | **human epidermal growth factor receptor 2 positive** |
| **CSF1R** | **colony stimulating factor 1 receptor** |
| **CCR2** | **CC motif chemokine receptor 2** |
| **CXCL-10** | **C-X-C motif chemokine ligand 10** |
| **NK** | **Natural killer** |
| **NKG2A** | |
| **HLA** | **Human Leukocyte antigen -E** |
| **ECM** | **extracellular matrix** |
| **TDLU** | **Terminal ductal lobular unit** |
| **TLRs** | **Toll like receptors** |
| **NF-$_K$B** | **Nuclear factor – KB** |
| **SNP** | **Single nucleotide polymorphism** |
| **FGFR1** | **fibroblast growth factor receptor 1** |

| | |
|---|---|
| **DAMP** | **danger-associated molecular pattern** |
| **TCGA** | **The Cancer Genome Atlas** |
| **mRNA** | **Messenger Ribonucleic acid** |
| **PI3K/AKT** | **Phosphoinositide 3-kinase- protein kinase B** |
| **LPS** | **Lipopolysaccharide** |
| **3'UTR** | **3'- untranslated region** |
| **PI3K-AKT-mTOR** | **Phosphoinositide 3-kinase- protein kinase B Mammalian target of rapamycin** |
| **mTOR** | **Mammalian target of rapamycin** |
| **PFS** | **progression-free survival** |
| **OS** | **overall survival** |
| **AI** | **Aromatase Inhibitor** |
| **Hsp90** | **heat shock protein 90** |
| **EGFR** | **Estimated Glomerular Filtration Rate** |
| **AKT** | **protein kinase B** |
| **PI3K** | **Phosphoinositide 3-kinase** |
| **DNA** | **Deoxy ribose nucleic acid** |
| **TNBC** | **Triple-negative breast cancer** |
| **NAC** | **Neoadjuvant Chemotherapy** |
| **BCS** | **Breast-conserving surgery** |
| **MUC1** | **Mucin 1** |
| **CA15-3** | **Cancer Antigen 15-3** |

| | |
|---|---|
| **CEA** | **carcinoembryonic antigen** |

## Summary

Two hundred specimens from woman with breast tumors (70 biopsies and 100 blood samples) , thirty other women (that had chemotherapy ) blood sample was taken from them . fifty blood sample from healthy women . Specimens involved biopsies (only from patients without chemotherapy) for bacterial cultivation , DNA extraction for polymorphism of TLR2. TLR4, IL1β and IL1α and evaluated immune markers (CA 15-3,TLR2 ,IL1α , IL1β). While blood (serum to determine systemic CA 15-3,TLR2 ,IL1α , IL1β) and whole blood for polymorphism .The age of women in this study ranging from (14-66) years in AL-Hilla-Teaching hospital , Imam Sadiq Hospital and Marjan hospital a period from September 2021 to October 2022.

The results appeared the most common breast tumor in woman at age group (30-45) years.

Identification of bacteria has been done by culture , biochemical test and Vitek 2 compact system ,In the present study the most common bacteria in malignant breast tumors was *Staphylococcus aureus* but in Benign breast tumors was *Klebsiella pneumonia* . A bacterial culture's output *Klebsiella pneumonia* was the most prevalent gram negative bacteria compared to gram positive bacteria.

In Enzyme Linked Immunosorbent Assay technique showed that the concentration of CA15-3 in patients was 106.307±28.881compared with control with mean was 62.802±17.629the result showed that concentration of CA 15-3 in patients with breast cancer before chemotherapy was significantly increased. The concentrations of IL1 α , IL1β and TLR2 in serum patients were ( 1.302±0.912, 5.599±3.550 and

9.953±4.606) significantly increased compare with control (0.617±0.240 3.640±1.996 and 6.774±3.855 )respectively

RFLP-PCR allele frequencies of IL-1 alpha -889 C>T in healthy and patients subjects allele CT in blood patients more affect than allele TT (odd ratio 1.28 , 0.72).but allele frequency CT in tissue patients more effect compared with allele frequency TT (1.00 , 0.33) , allele frequency TT in malignant and benign was more effect than allele CT (0.90 , 0.70) respectively , but in chemotherapy patients allele frequency TT was more effect compared with allele CT (1.50 , 0.91) respectively . in tissue of malignant and benign whereas patients with CT more affected by breast tumors comparison with patients having genotype TT(Odd ratio 1.16, 0.98).

RFLP-PCR allele frequencies IL-1 beta C31 T in blood patients with genotype TT were affected by breast tumors approximately comparison with patients having genotype CT (odd ratio = 0.93 and 0.59 ). Allele frequency of IL-1 beta C31 T in tissue , patients with genotype CT were affected by breast tumors approximately one time comparison with patients having genotype TT (odd ratio = 0.62 and 0.58) .but Allele frequency of IL-1 beta C31 T chemotherapy patients with genotype TT were affected by breast tumors approximately one time comparison with patients having genotype CT (odd ratio = 1.01 and 0.76) . Allele frequency of IL-1 beta C31 T in benign and malignant patients with TT , CT more affected by breast tumors comparison with patients having genotype CT(Odd ratio 0.31 , 0.31). In tissue of benign and malignant whereas patients with CT more affected by breast tumors comparison with patients having genotype TT(Odd ratio 0.38, 0.26).

TLR2 Asp 299 Gly genotyping PCR the result of sequencing show that deletion, insertion and trans version in G,C,T,A nucleotide but deletion in G and C nucleotide patients' blood was higher compared with healthy control . Allele frequency of TLR4 3725 in blood patients with genotype CC were affected by breast tumors approximately one time comparison with patients having genotype GC (odd ratio = 1.49 and 1.01).

In tissue patients with genotype CC were affected by breast tumors approximately one time comparison with patients having genotype GC (odd ratio = 1.49 and 1.01) . , in chemotherapy patients with genotype GC were affected by breast tumors approximately one time comparison with patients having genotype CC(odd ratio = 0.66 and 0.25) . in blood benign and malignant patients with GC more affected by breast tumors comparison with patients having genotype CC(Odd ratio 2.77 , 0.55).in tissue benign and malignant patients with CC more affected by breast tumors comparison with patients having genotype GC(Odd ratio 3.20 , 2.24).

## 1-1 Introduction

Multiple disorders are included under breast diseases. Noncancerous breast conditions predominate. Some of these lesions are clinically unremarkable and just required minor treatment. But some symptoms might have clinical significance and draw the attention of the patient and the treating physician, especially if they continue to persist (Alamri1 *et al*.,2020).

The majority of breast cancers (BC) are age-related, and researchers are trying to figure out what cellular and molecular changes in breast tissue take place as people age and make women more prone to developing cancer. The immune system is crucial for the growth of the mammary glands and has a significant impact on the development of BC. (Zirbes *et al*.,2021).

The microbial spectrum in breast tissues from breast cancer patients differs dramatically from that of normal breast tissue. Proteobacteria are the most prevalent type of them in healthy breast tissue (Mani, 2017). *Proteobacteria* , *Firmicutes* , *Escherichia coli* , *Methylobacterium radiotolerans* , *Mycobacterium fortuitum* , *Mycobacterium phle i*, *Corynebacterium* , *Staphylococcus* , *Actinomyces* , *Propionibacteriaceae* , *Propionicimonas* , *Micrococcaceae* , *Caulobacteraceae* , *Rhodobacteraceae* , *Nocardi In addition to others*, *Arcanobacterium haemolyticum* , *Peptoniphilus indolicus* , *Prevotella nigrescens* , *Propiniobacterium jensenii* , *Capnocytophaga canimorsus* , *Fusobacterium* , *Atopobium* , *Gluconacetobacter* , *Hydrogenophaga* , and others have been identified (Meng *et al* ., 2018 ).

*Lactococcus and Streptococcus are more prevalent in healthy tissues than BC.. Additionally, Lactococcus stimulates NKT cell activation, resulting in cellular immunity that inhibits the growth of cancer* (Kurath-Koller et al.,2017). Prevotella was also found in higher concentrations in healthy tissues, according to Urbaniak et al. These microorganisms have the capacity to create SCFA, which has antitumor effects (Urbaniak et al.,2016). Breast cancer has one of the highest mortality rates in the world and is the most typical type of advancing cancer in women (Yazdani-Charati et al.) the most prevalent kind of cancer and the main reason why women die in Iraq is breast cancer. 2017 (Abdalzahra and Ali) cases of Iraq saw a rise in breast cancer from 26.6 per 100,000 people in 2000 to 31.5 per 100,000 people in 2009.according to studies. In 2020, Zuhair et al. Additionally, it was shown that the prevalence of old age in Iraq was lower than that in Jordan and Kuwait and higher than that in Bahrain, Saudi Arabia, Turkey, and Iran. (Ferlay et al.,2015).

immune cells, including certain epithelial cells and macrophages, dendritic cells, mast cells, and eosinophils typically express toll-like receptors (TLRs) (Ayala-Cuellar et al.,2019). Endogenous inhibitors of TLR2 and TLR4 activation are soluble Toll-like receptors (sTLR) 2 and 4. So, it is possible that sTLR2 and sTLR4 could serve as indicators of breast cancer vulnerability (El-Kharashy , 2021).

The inflammatory One of the cytokine family members is interleukin-1 (IL-1). and is involved in both innate and adaptive immunity as well as wound healing. The IL-1 family includes the important, closely related IL-1 and IL-1., support tumorigenic characteristics and aid in cancer therapy resistance. Additionally, IL-1 controls intracellular signaling and the hormone receptor expression of

BCa cells in a way that gives tumor cells an advantage in growth and enables BCa cells to resist treatment (Diep *et al*.,2022).

Cytokine gene single nucleotide polymorphisms (SNPs) have been shown to change their expressions or functions in BC patients. The molecular makeup and purpose of IL-1 have been investigated recently. Its genetic polymorphism might have an impact on the IL-1 gene's transcription and expression. Furthermore, it has a tight connection to a number of disorders (Wang et al.,2019). The three SNPs (rs16944, rs1143634, and rs1143627) in IL-1 and breast cancer risk have been the subject of numerous studies to date (Zuo *et al*.,2018).

Recently, there has been a lot of interest in TLR4 polymorphisms in people with breast cancer. the association between breast cancer risk and the TLR4 +3725G/C polymorphism, however, has received relatively little research (Zamzam *et al* .,2019).

In order to fulfill the goal of the current study, which was to examine the relationship between bacterial breast cancers and IL1 polymorphism, TLR4 polymorphism, and TLR2 polymorphism, the following axes of study were used:

1- Breast tissue bacterial isolation and identification

2- Use RFLP-PCR to find the SNP of IL1 α,β TLR4, and TLR2

3- Use ELISA to calculate the levels of IL1 α,β and TLR2 in systemic (serum) and mucosal tissue.

4- Use an ELISA to calculate the patient's serum CA 15-3 levels.

5- To prove the type of SNP and to sequence any IL1 α,β, TLR4, and TLR2 SNPs that are discovered.

## 2-1 Breast disease

Mostly females are reported to have breast lesionsBenign breast illness, which is often 10 times more prevalent than The most frequently reported cause of female breast issues is breast cancer (Hatim *et al*.,2017).

The primary factor causing that worry is the potential for breast cancer, which is typically asymptomatic in the early stages when it is treatable (Aslam *et al*.,2013).

Benign breast disorders comprise a wide range of histological patterns that can be divided into proliferative and non-proliferative breast lesions as well as proliferative breast lesions with and without unusual features (Okoth *et al*.,2013) Certain benign breast lesions have been discovered to be a significant breast cancer risk factor (Clavel-Chapelon , Gerber , 2002) In western nations, women with benign proliferative or atypical breast lesions have a twofold more danger of development of breast cancer (Coriaty Nelson *et al*.,2002).

## 2-2 Anatomy of Breast

Adipose, glandular, and fibrous tissue of various sorts are housed The lobes are supported by a stroma that is fatty and fibrous. Figure. are all involved in lymphatic drainage, which largely occurs through the axillary lymph nodes figure (2-1) (Khan and Sajjad , 2021).

Males and children both have breast tissue, however due to hormonal changes that occur throughout puberty, females of reproductive age have more developed breast tissue. Following menopause, breast

tissues progressively involute; the glandular tissue atrophies due to the drop in estrogen levels in the blood and is primarily replaced by fatty tissue. Changes in hormone levels can affect breast tissues and the majority of breast diseases (Khan and Sajjad , 2021).

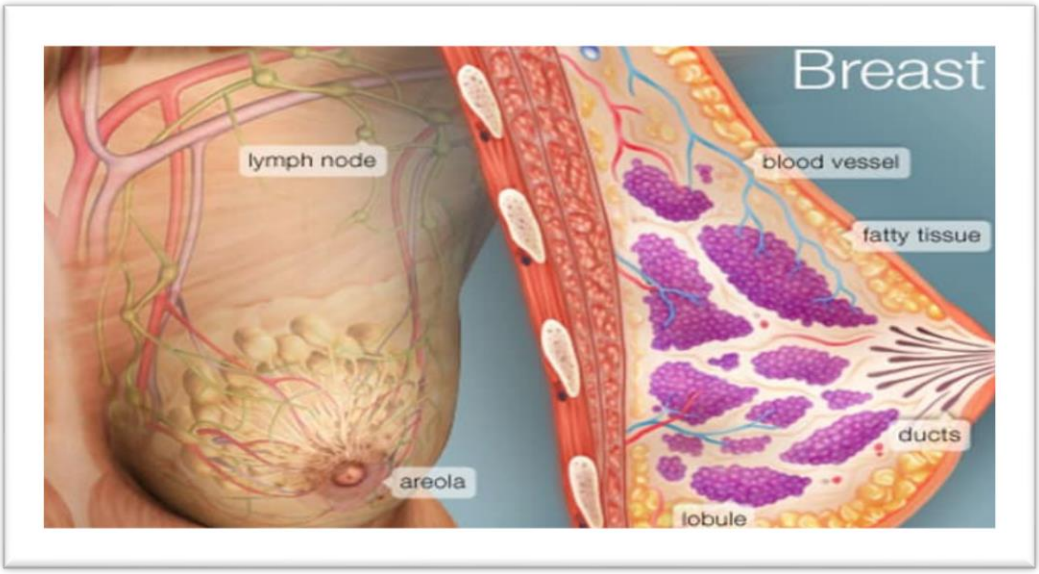



**Figure (2-1) Human Breast anatomy (Carol DerSarkissian, 2021)**

## 2-3 Risk Factors

Exposure to estrogen in excess is the primary contributor to breast cancer risk. Therefore, it is imperative to inquire about their lifetime estrogen exposure from every patient who has a new breast tumor. (Akram *et al*.,2017) Male patients should be questioned about any history of orchitis/epididymitis flare-ups, finasteride use, testosterone use, or Klinefelter syndrome diagnosis (Yalaza *et al*.,2016).

### 2-3-1Genetic mutation

A nuclear phosphoprotein that is produced by the BRCA1/2 DNA repairs-related BRCA1 gene product (Savaridas *et al*.,2017). With a more prevalence of cancer risk at younger ages, women with BRCA1 mutations had lifetime breast cancer risks of up to 80%. Prostate cancer is more common in men who carry BRCA pathogenic variants, while breast cancer is around 6% more frequent in women who carry BRCA2 pathogenic variations across their lifetimes (Ribeiro Pereira *et al*.,2017) .

### 2-3-2 Body mass

Breast cancer can develop and spread due to environmental causes as well (Lambertini *et al*.,2017). Having a high BMI (body mass index) could potentially worsen outcomes for people with a history Breast cancer risk increases with post-menopausal status. Breast cancer risk is determined by breast density. According to a research, women are 4-6 times more likely than men to acquire breast cancer. higher if her breast tissue is between 60 and 75 percent thick whose breasts don't contain any dense tissue. Even while an increase in BMI is

## 2-4 Types of breast tumor

Breast ultrasound pictures' morphological and textural characteristics are frequently utilized to distinguish between benign and malignant cancers. The simple method is to manually analyze the texture and morphological aspects in pictures and rely on highly qualified and experienced radiologists to determine whether a tumor is benign or malignant (Steifer and Lewandowski, 2019). To avoid the subjectivity of manually analyzing ultrasound images, another simple method is to train

## 2-4-1 Benign breast tumor

Up to 50% of premenopausal women have fibrocystic alterations of the breast, commonly known as fibrocystic mastopathy, which is a fairly prevalent benign condition (Guray and Sahin , 2006). Numerous histopathologic alterations, including cyst development, sclerosing adenosis, duct ectasia, apocrine metaplasia, papillomatosis, and stromal fibrosis, are included in fibrocystic mastopathy (Orr and Kelley , 2016).

These changes are frequently accompanied by epithelial modifications including benign columnar cells lesion (CCL), flates epithelial atypical, and typical ductal hyperplasia (UDH) (Brunner *et al*.,2014). Classic fibrocystic alterations, whether they include or exclude epithelial changes, are not thought to be precancerous lesions because invasive carcinoma only occurs in a very tiny proportion of these women. Although this risk is independent of other key epidemiologic the risk breast cancer factors, women with fibrocystic breast disease have a markedly enhanced occurrence of breast cancer (approximately 1.5–2 times that of the general population). This risk is especially obvious in women over 50 (Zendehdel *et al*.,2018).

Due to the frequent occurrence of these alterations in the general population, the absolute risk is large even though the relative risk appears to be low. However, it is still challenging to predict which patients will acquire invasive cancer, which inspires research targeted at identifying the molecular mechanisms at work in these first phases of cancer formation (Brunner *et al*.,2014) The most frequent benign breast tumor in young women is a fibroadenoma. It is distinguished histologically as a

biphasic tumor having both stromal and epithelial components (Lee *et al*.,2015) .

Although fibroadenomas can develop at any age, they are most commonly found in young girls between the ages of 20 and 30 and have a low occurrence after the fourth decade of life (Chen *et al*.,2018) Although the specific cause of fibroadenoma is unknown, it is likely that elevated estrogen levels and increased estrogen receptor sensitivity are to blame for the tumor's dramatic development alterations (Celik *et al*.,2017s).

Histologically, breast lumps can be divided into proliferative and non-proliferative lesions. Fibro adenomas, intraductal papillomas, and benign phyllodes tumors are examples of proliferative lesions. Proliferative or non-proliferative fibrocystic alterations are possible, although in 70% of cases, are detected (Guray and Aysegul , 2006). Benign tumors are generally not hazardous. However, since a benign breast tumor is one of the precursors to breast cancer, we must be aware of this (Falco *et al*.,2019).

The cause of benign breast tumors is yet unknown. However, hormonal changes in a woman's body are directly tied to changes in her breasts (National Cancer Institute , 2015). Both hormones are present in hormonal birth control methods including oral contraceptives. Oral contraceptives may or may not raise the risk of benign breast cancers (Sihombing *et al.*, 2015). This is still up for debate. Between 25% and 50% of adult women have benign breast illness, which is frequently more

prevalent and accounts for 3% of female patients' visits with general practitioners ( Stachs *et al*.,2019).

**2**

### 2-4-2-1-3 Metaplastic carcinoma

This histological subtype, which affects women in the post-menopausal stages and makes up 1% of all cases, can be identified by the preponderance of metaplastic differentiation (Sinn and Kreipe , 2013). This particular tumor type has an aggressive biological action, and lymph nodes are frequently affected by it (Schwartz *et al*.,2013) .

### 2-4-2-1-4 Apocrine

acquire this subtype, which often has a high histological grade and a bad prognosis and affects persons of all ages (Vranic *et al*.,2013).

### 2-4-2-1-5 Mucinous carcinoma

2% of all newly diagnosed instances of breast cancer are of this specific form, which is defined as colloid, gelatinous, mucous, and mucoid carcinoma (Akram *et al*.,2017). This subtype frequently affects women over the age of 60 and has been linked to a positive outcome (Marrazzo *et al*.,2020).

### 2-4-2-1-6 Cribriform carcinoma

A distinct subtype with a good prognosis that typically affects people around the age of 50 and accounts for 1% to 3.5% of all breast

cancer cases frequently affects people in this age group (Vuong *et al*., 2014). In cribriform carcinoma, there is seldom any proof of either local or distant metastases (Makki , 2015) .

## 2-5 Histopathological types of breast cancer

Prior research on the morphology of breast cancer helped to determine whether the tumor was restricted to the breast's epithelial layer or had spread to the stroma around it and whether it had originated in the mammary ducts or lobes (Vuong *et al*., 2014). However, in histopathological practice, rather than the tumor's exact location in the mammary tissue, In addition to its subclassifications, factors such as the tumor's cell type features, cell quantity, The tumor's immunohistochemical profile, architectural features, type and location of secretion, and architecture determine whether it is ductal or lobular. (Makki , 2015 ; Nounou *et al*.,2015).

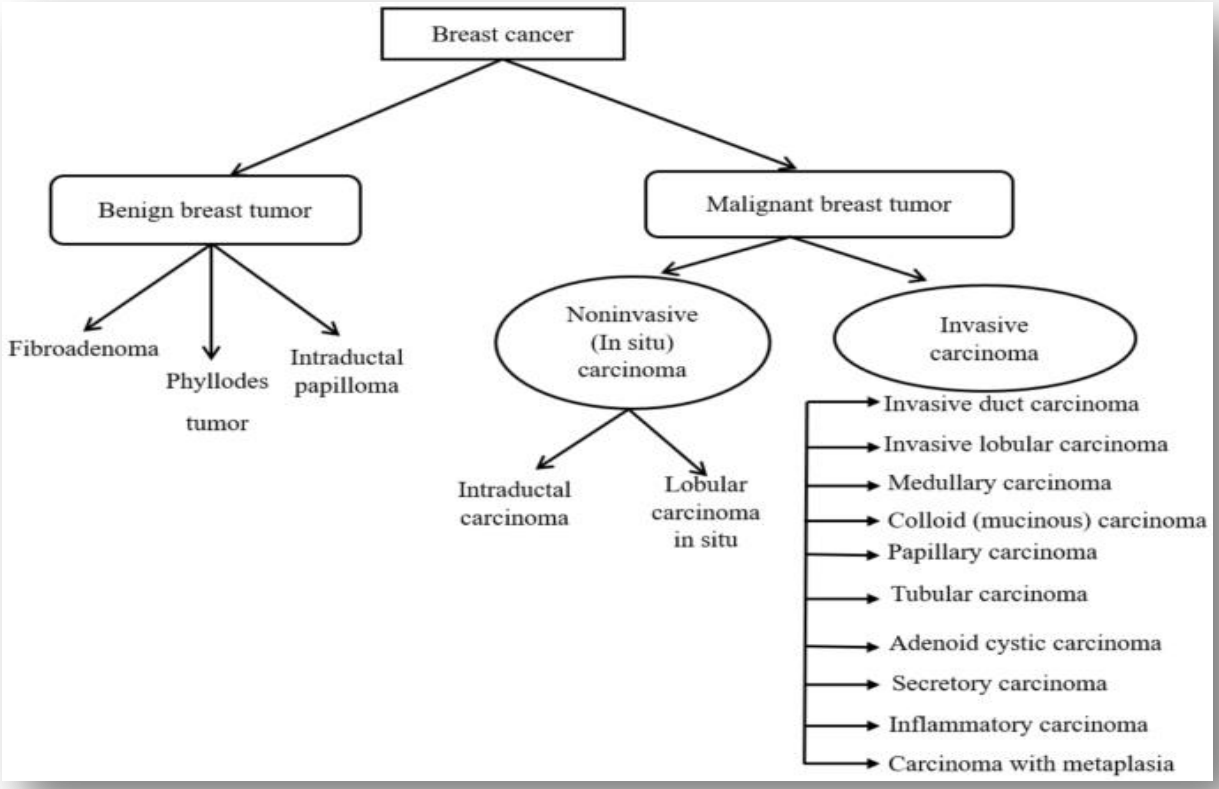



**Figure (2-2) Histopathological types of breast cancer (Mohan , 2010)**

## 2-6 Microbiome and breast disease

al colonization, immune system maturation, and metabolic development during nursing. The existence of butyrate-producing bacteria in human milk, including Roseburia species, Coprococcus species, and Faecalibacterium prausnitzii, is an illustration of this. These species can alter the risk of cancer, allergy conditions, and childhood obesity(Prentice *et al*., 2019; Zhang *et al*., 2020).

## 2-7 Antitumor Immunity

A microenvironment that includes a variety of marrow-Hematogenous and lymphatic vascular cells, immune cells, derived support cells (fibroblasts, adipocytes, etc.), a nest of malignant tumor cells (ECM). aggressive and/or metastatic transformation of neoplastic growth is influenced by this microenvironment (Hanahan and Weinberg ,2011 ; Hanahan and Coussens ,2012).

TCRs undergo genetic rearrangement throughout T cell development, which makes it possible for adults to recognize a variety of processed Ags. A growing body of research indicates that the production of suppressive cytokines like interleukin (IL)-10 renders T lymphocytes specific for tumor antigens in solid tumors tolerant of the antigens or prevents them from killing tumor cells (O'Garra and Saraiva,2010) are both arginose. (Crittenden *et al*.,2014) .

## 2-8 Immunity of breast tumor

Healthy breast tissue contains immune cells from the lymphoid (T lymphocytes and B lymphocytes) and myeloid (monocytes, macrophages, and dendritic cells) lineages. (Degnim *et al*.,2014). At various stages of breast carcinogenesis, There is a high prevalence of inflammation related to cancer, including cancer extrinsic inflammation and cancer intrinsic inflammation (Lim *et al*.,2018 ; Comen *et al*.,2018). Typically, cancer-intrinsic inflammation is caused

Leukocytes are drawn in and moved to the site of the tumour, where they are later activated as the tumor develops. However, infections caused by germs or viruses, obesity, excessive alcohol intake, smoking,

hormone therapy, autoimmune diseases, excessive radiation exposure, etc. are all connected with cancer-extrinsic inflammation (Comen *et al*., 2018). Leukocytes act as a barrier and selective pressure for the growth of tumors by mounting antitumor immune responses (Kroemer *et al*.,2015 ).

Adaptive immunity is triggered by Innate immune responses, which are the initial line of defense against infections and malignancies and do not need antigens to activate them. Although they are scarce in normal breasts, CD45 leukocytes can be seen in the stroma and mammary ducts (Towe *et al*.,2019).

in HER2-positive breast carcinoma models where macrophages recruited by tumor-derived CCL2 do so. In DCIS, CD68 macrophage are seen in ducts close to cancer cells with reduced levels of E-cadherin, suggesting that they may have a similar role in encouraging progression (Linde *et al*.,2018).

Innate immune cells called natural killer cells participate in the initial immune response to malignancies (Laskarin *et al*.,2010; Kamiya *et al*.,2019) NK cells are renowned for their function in tumor immunosuppression and their response to inflammatory stimuli. In humans, NK cells are characterized as CD56+ CD16+CD3- phenotypically (Laskarin *et al*.,2010; Gulic *et al*.,2018). The interaction

between the NK cell's array of activating and inhibitory receptors determines how active they are (Laskarin *et al*.,2010 Gulic *et al*.,2018)

Interferon, chemokines, Granulocyte macrophage colony-stimulating factor (CCL1, CCL2, CCL3, CCL4, CCL5, and CXCL8), tumor necrosis factor are examples of proinflammatory cytokines that NK cells secrete after activation. These cytokines can influence how other innate and adaptive immune cells function (Kamiya *et al*.,2019; Silva *et al*.,2013; Mamessier *et al*.,2011). One of the NK inhibitory receptors, CD94/NK group 2 member A (NKG2A), binds to minimally polymorphic nonclassical HLA class I molecules, such as the overexpressed HLA-E seen in many solid malignancies (Kamiya *et al*.,2019 ; Gulic *et al*.,2018)

Additionally, according to Gulic *et al*. (2018), HLA-E expression may be a key regulatory strategy for cancers to evade immune monitoring. The therapy of invasive breast tumors with lower levels of malignant cellular immunogenicity is established by the use of tolerogenic NK cells (Mamessier *et al*., 2011). Cellular Ig-like receptors or NKG2A serve as critical receptors for NK cells' tolerogenic capabilities, which are crucial for recognizing breast carcinoma cells (Mamessier *et al*.,2011 ; Tu *et al*.,2017).

Figure shows The relationship between the ductal structure and the location of immune cells in a healthy breast (2-3). The acini, which is the primary component of the breast ductal system, drains into the intralobular and eventually extralobular ducts, which together make up lobular terminal unit of the duct (TDLU). A second layer of cuboidal epithelium or pseudostratified columnar epithelium lines the main ducts

and extralobular terminal ducts., respectively, while cuboidal epithelium lines the intralobular terminal ducts. It is believed that the TDLU is where breast cancer usually first manifests itself (Yang *et al.*,2016).

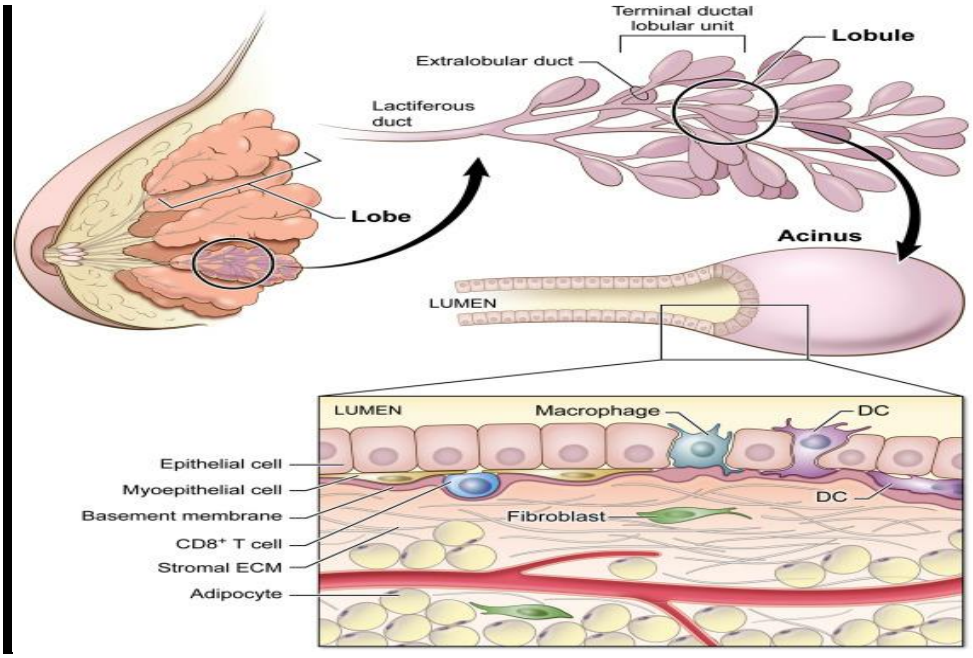



**Figure (2-3)Nature and distribution of Immune cells in normal breast tissue.**

Abundant immune cells, Lymphocytes and myeloid-derived cells can be seen in healthy breast tissue. Although they can sometimes be found in the stroma, The ductal epithelium (intraepithelial) is where immune cells are most commonly found (Not Depicted). Immune cells and the epithelium can interact with a variety of different cell types and structures in the stromal extracellular matrix (ECM) (Degnim *et al.*,2014)

## 2-9 Toll like receptor 2

The well-conserved pattern-recognition receptors known as toll-like receptors (TLRs) are mostly expressed in human epithelial and immunological cells (Mifsud *et al*.,2014 ; Brubaker *et al*.,2015). TLRs' primary purpose is to encourage the production and release of inflammatory cytokines and chemokines, which sets off the inflammatory response (Palm *et al*.,2015 ; Johnston and Corr, 2016) .

Although the relationship between TLRs and breast cancer has not been completely studied, earlier research revealed that there may be a significant connection. Nuclear factor-B (NF-B), transforming growth factor (TGF), vascular endothelial growth factor, and matrix metalloproteinase 9 are all upregulated as a result of TLR2 activation (Xie *et al*.,2009).

TLR2 has an interesting property that makes it a possible therapeutic target in highly invasive breast cancer: activation of the receptor was discovered to boost the invasive potential through NF-B signaling (Xie *et al*.,2009). Through NF-B signaling, it has been shown that TLR2 stimulation on the surface of breast cancer cells increases the disease's propensity for invasion (Xie *et al*.,2009). Additionally, new research indicates that TLR2 signaling might help tumor cells elude immune system attack and immunological surveillance (Huang *et al*.,2008).

## 2-10 IL1 alpha and IL1 beta

Through inflammation in the tumor microenvironment, interleukin 1 is hypothesized to play a significant role in the invasiveness, development, and metastasis of cancer (Wu *et al*., 2018)). Additionally,

according to Wu et al. (2016), IL-1R and IL-1 variants have also been linked to the development and beginning of breast tumors (Snoussi *et al*.,2005) .

One hypothesis is that IL-1 stimulates the production of IL-6 via the transglutaminase/NF-B pathway. The aggressiveness of breast cancer cells of the luminal type rises as a result. Anti-IL-1 or anti-IL-6 medications can be used to inhibit this (Oh *et al*.,20016). The fibroblast growth factor receptor 1 (FGFR1)-induced mouse mammary cancer model has been used to describe another mechanism. It links early-stage mammary tumors to COX-2 production that is mediated by IL-1 (Reed *et al*.,2009).

## 2-11 Molecular study of IL1beta and alpha in breast disease

The transcription factor IL-1 can be located in the nucleus, where it works to regulate both the growth of healthy cells and the growth of cancerous cells. However, IL-1 translocates into the cytosol and is released into the extracellular space to act as a "alarmin" when cells undergo cell death, such as necrosis. (Rider *et al*.,2013 ; Dinarello , 2009).

Contrarily, In order to activate IL1B gene transcription is made possible by inflammasome complexes and inflammatory caspases, which convert pro-IL-1 into mature IL-1 the production and processing of IL-1 require two signals (Schroder and Tschopp , 2010).The influence of IL-1

on the onset or course of cancer can be used to gauge its significance. This assessment can be carried out by evaluating the expression of IL1B mRNA, IL-1 protein, or gene polymorphisms that may affect it. Using the cancer genome atlas (TCGA) database, it was discovered that breast cancer patients with higher levels of IL1B mRNA expression than those with lower levels had a better prognosis (Martínez-Reza *et al*.,2019).

Variations in IL-1 expression may be related to IL1 gene polymorphisms. IL-1β-31 (rs1143627) (rs1143627) T alleles are linked to higher IL1B expression. Breast cancer risk was greater when the T/T genotype was present (Akisik and Dalay , 2009 ; Eras *et al*.,2019).

## 2-12 Polymorphism of TLR2

According to Zhu *et al*. (2013), TLR2 polymorphisms are linked to an increased risk of BCa and have a role in immunomodulation in BCa (Chow *et al* .,2014). TLR2 has a significant role as a proto-oncogene by being elevated in the majority of cancers and closely linked to tumor metastasis (Wang *et al*.,2019 ; Liu *et al*.,2016). TLR2 stimulates the PI3K/AKT signaling pathway, which aids in the growth, migration, and invasion of tumor cells in colorectal cancer (Liu *et al*.,2018).

The clinical relevance of TLR2 in BCa is still unknown, though. TLR2 agonists improve the effectiveness of HER2-targeted monoclonal antibody therapy, according to research by Lu et al (Lu *et al*.,2011). In the promoter of the TLR2 gene, a polymorphism that results in a 22-bp nucleotide deletion (196 to 174 del) has been discovered through genetic research. The promoter's functionality may be dramatically altered by this alteration, most likely resulting in less TLR2 transcription (Noguchi *et al*.,2004).

## 2-13 Polymorphism of TLR4

As a result, the transcription factor NF-B is activated, which triggers the expression of interferons, adhesion molecules, growth factors, inflammatory cytokines, and chemokines that assist control immune system activity (Garca Bueno *et al*., 2016). Chronic inflammatory conditions may result in carcinogenesis if TLR4-induced inflammatory signaling is persistently activated (Basith *et al*.,2012 ) Various human malignancies have been reported to express TLR4 (Gambara *et al*.,2013) .

According to one study, TLR4 expression on tumor cells was found in 63% of breast cancer patients, and the amount of expression was inversely connected with survival (Bhatelia *et al*.,2014). The normal cellular immune response can be disrupted by single nucleotide polymorphisms (SNPs), which also increases the risk of cancer and chronic inflammation. SNPs can change ligand binding and the equilibrium between pro- and anti-inflammatory cytokines (Shen *et al*.,2013) .

Numerous research have looked into the relationship between TLR4 SNPs and cancer risk, however few have shown evidence of a TLR4 polymorphism and breast cancer association (Zhang *et al*.,2013) The TLR4 gene's 3'-untranslated region (3'UTR) is home to the TLR4 +3725G/C polymorphism, a single nucleotide variation that has been linked to both cancer and inflammation (Hishida *et al*.,2009)

## 2-15 CA15-3 and breast tumors

A chemical that is released by both cancer cells and healthy cells is a tumor marker. When it comes to cancer activity, it manifests at much higher levels. It is a little intrusive, somewhat inexpensive method of diagnosis. However, for a technique of diagnosis to be dependable, it must exhibit great sensitivity for tumor diagnosis even in its early stages (kilpatrick and lind ,2009) Following chemotherapy and radiotherapy, tumor markers are also helpful in determining the prognosis for cancer, therefore they could serve as a foundation for modifying ongoing management (amayo and kuria,2009).

The MUC1 protein is known to be upregulated in malignant breast tumors, which is the rationale for the CA 15-3 protein's potential as a breast cancer biomarker. In several trials, blood CA 15-3 levels were used as a screening tool for breast cancer and other malignant tumors. However, numerous false positive results have been documented, such as in the cases of smoking and benign breast and liver disorders (bahrami-ahmadi *et al.*,2012) .

It is suggested that CA15-3 and CEA can be used as biomarkers to help with the diagnosis of breast cancer recurrence patients. Darlix et al. discovered that the CA 15-3 in patients with metastatic breast cancer is an independent predictive factor (shao *et al*.,2015).

## Materials and methods

### 3-1 Materials

### 3-1-1-: Equipment for Laboratories

The Table provides an illustration of the instruments utilized in this investigation table (3-1)

**Table (3-1): Equipment and instruments for laboratories**

| NO. | Instrument | Company | Country origin |
|---|---|---|---|
| 1 | Autoclave | Tripod | UK |
| 2 | Beakers and flask | Steriline | UK |
| 3 | Bench centrifuge | Memmert | Germany |
| 4 | Burner | Amal | Turkey |
| 5 | Different size of tips | Meheco | China |
| 6 | Digital camera | Sony | Japan |
| 7 | Electric sensitive balance | Denver | USA |
| 8 | ELISA reader | Biotech | USA |
| 9 | Eppendorf tubes | Eppendorf | Germany |
| 10 | Gel documentation system | Vilber | France |
| 11 | Gel electrophoresis system | Cleaver Scientific | UK |
| 12 | High speed centrifuge | Hettich | Germany |
| 13 | Hood | Bio LAB | Korea |
| 14 | Incubator | Selecta | Spain |
| 15 | Light microscope | Olympus | Japan |

| | | | |
|---|---|---|---|
| 16 | Loop | Shndon | UK |
| 17 | Microcenterfuge tubes | Biobasic | Canada |
| 18 | Micropipettes | Capp | (Denmark) |
| 19 | Oven | Olympus | Japan |
| 20 | Para film | BDH | Ergland |
| 21 | PCR Thermal cycler | Techne | UK |
| 22 | Petri dish | Sterilin | England |
| 23 | Plain tubes | DMD-DISPO | Syria |
| 24 | Refrigerator | Kiriazi | Egypt |
| 25 | Slide | Sail Brand | China |
| 26 | Sterile hypodermic syringe | EL-dawlia ico | Egypt |
| 27 | Sterilize Swab | ATACO | Brand |
| 28 | Surgical Blades | | |
| 29 | UV transilluminator | ATTA | Korea |
| 30 | Vitek 2 system | Biomerieux | France |
| 31 | Vortex mixer | Griffin | Germany |

### 3-1-2 : Chemical materials

Chemical components utilized in the investigation are represented in Table (3-2), including reagents, stains, and solutions

**Table 3-2: Chemical substances**

| NO. | Types of chemical | Company /origin |
|---|---|---|
| 1 | 6X DNA Loading buffer Blue | Eurx (Poland) |
| 2 | Absolute Ethanol | Scharlau – Spain |
| 3 | Agarose | Condalab (spain) |
| 4 | Ethanol 99% | Merck-England |
| 5 | Ethidium bromide | Bioneer – korea |
| 6 | Glycerol ($C_3H_8O_3$) | Merck-England |
| 7 | Gram stain set | BDH, England |
| 8 | Ladder 100bp | **Bioner** |
| 9 | Ladder 50 bp | |
| 10 | Nuclease free water | Bioneer – india |
| 11 | Polyacrymid gell | |
| 12 | ,Kovac's reagent, sterile urea, -Naphthol, KOH | Sigma, USA |
| 13 | TBE buffer 10 x | INTRON - korea |

### 3-1-3: media for Culture

Table provides examples of the cultural media that were employed in this study table (3-3):-

**Table (3-3): Culture media that used in this study**

| NO. | Type of media | Manufacturing company | Origin |
|---|---|---|---|
| 1 | Blood agar base | Himediaf | Indiah |
| 2 | Brain heart infusion broth | | |
| 3 | Eosin methylene blue | | |
| 4 | agars of Kligler Iron | Diffco-Michigan | USA |
| 5 | MacConkey agar | Himediat | Indian |
| 6 | Mannitol salt agar | | |
| 7 | MR_VP broth | | |
| 8 | Muller Hinton agar | | |
| 9 | water of Peptone | | |
| 10 | agar Simmon citrate | Diffco-Michigan | USA |

### 3-1-4: kits Commercial

Tables 3–4 show examples of the commercial kits that were used in the investigation.

**.Table (3-4): Commercially available kits were used in this study**

| NO. | Type of Kit | Company / Country |
|---|---|---|
| 1 | DNA G spin total | Korea |
| 2 | extraction Kit of DNA | Favorgen |
| 3 | ladder of DNA | SIMGEN |
| 4 | master mix Green | SIMGEN |
| 5 | Primers | |
| 6 | Interleukin 1 Alpha | Elabscience / U.S.A |
| 7 | Interleukin 1 Beta | Elabscience / U.S.A |
| 8 | Toll like receptor 2 | Elabscience / U.S.A |
| 9 | CA 15-3 | Elabscience / U.S.A |

## 3-2 Molecular kits and reagents used in genetic polymorphism detection

### 3-2-1 DNA extraction kits for human tissue (G spin total DNA )

Table lists the kits and reagents used in this investigation along with their manufacturers and countries of origin ( 3-5 ).

**Table (3-5 ) DNA extraction kit**

| Kit | Company | Country |
|---|---|---|
| G-spin ™ total DNA extraction kit | iNtRON | Korea |
| BL Buffer | | |
| WA Buffer | | |
| WB Buffer | | |
| CE Buffer | | |
| Column Spin | | |
| 2ml of Collection tube | | |
| 11mg/ml of Proteinase k | | |
| 9 mg/ml of RNase | | |

### 3-2-2 DNA extraction kits for human $RBC_S$ (FAVROGEN) .

Using the Favorgen kit, human genomic DNA was isolated from frozen white blood cells (WBCs).

**Table (3-6) DNA extraction kit for frozen blood and bacteria**

| Cat.No. / Preps | FABG K 100 (100 PREPS) ml | FABG K 300 (300 PREPS) |
|---|---|---|
| **Buffer lysis RBC** | 135 | 405 ml |
| **FATG Buffer** | 30 | 75 ml |
| **Buffer FABG** | 40 | 100 ml |
| **Buffer W1** | 45 | 130 ml |
| **Buffer concentrated wash** | 25 | 50 ml |
| **buffer Elutions** | 30 | 75ml |
| **FABG of column** | 100 pcs | 300 pcs |
| **collection tube of 2 ml** | 100 pcs2 | 100 pcs |

**When first opening, 100 or 200 ml (96-100%) of ethanol should be * ..added to the wash buffer**

### 3-2-3 Primers and DNA marker

The primers employed in the current study's DNA marker, a 100 bp and 50 bp ladder from the Chinese business SIMGEN, are listed in the table (3–7) below.

**Table ( 3-7 ) Primers for amplification of IL1 β , IL1α , TLR2 and TLR4 gene**

| Primers | | Sequences | Amplicon size bp | References |
|---|---|---|---|---|
| IL-1β-31T | **F** | 5′-AGA AGC TTC CAC CAA TAC T-3′ | 240 | Akisik and Dalay ,2007 |
| | **R** | 5′-TAG CAC CTA GTT GTA AGG A-3′ | | |
| IL1 α -889 C>T | **F** | 5’-TTACATATGAGCCTTCCATG-3 | 108 | Emiroğulları *et al*.,2018 |
| | **R** | 5’AAGCTTGTTCTACCACCTGAACTAGGC-3’ | | |
| TLR4 +3725G/C | **F** | 5′ACAAGTGATGTTTGATGGAC-3' | 361 | Zamzam *et al*.,2019 |
| | **R** | 5′ GCCATTCTACCTGGTATAAG-3' | | |
| TLR2 Asp299Gly | **F** | 5 -CACGGAGGCAGCGAGAAA-3 | 286 | Theodoropoulos *et al*.,2012 |
| | **R** | 5 –CTGGGCCGTGCAAAGAAG-3 | | |

### 3-2-4 Restriction enzyme

In the RFLP-PCR test, restriction enzymes from the company and nation .(listed in table were utilized (3-8

**Table (3-8 ) Restriction enzymes**

| Gene | Restriction enzyme | Company / country |
|---|---|---|
| IL1 α -889 C>T | *Nco I* | Thremofisher |
| IL1 β C31T | *AluI* | Thremofisher |
| TLR2 Asp299Gly | | |
| TLR4 +3725G/C | *Ear I.* | Thremofisher |

### 3-3 Enzyme –linked Immunosorbent Assay (ELISA) kits

### 3-3-1 Human IL1β , IL1α , TLR4 and TLR2 ELISA (Elabscience, china )

Human Enzyme-Linked Immunosorbent Assay (ELISA) kit was used to measure the levels of IL-1, IL-1, TLR4 and TLR2 in serum and saliva. The kit's are components mentioned the tables ( 3-9)

**Table ( 3-9 ) contents of human IL1β ELISA kit**

| Reagents | Specifications | Storage temperature |
|---|---|---|
| Micro ELISA plate | 8 well × 12 strips | 4ºc / -20ºc |
| Reference standard for IL1β | 2 vials | 4ºc / -20ºc |
| Reference standard & specimen diluents | 20mL 1 vials | 4ºc |

| | | |
|---|---|---|
| for IL1β | | |
| Concentrated biotinylated detection Ab for IL1β | 120 µL 1 vial | -20ºc |
| IL1β of biotinylated detection Ab | 10 mL 1 vial | 4ºc |
| IL1β Concentrated HRP conjugate | 120 µL 1 vial | 4ºc (shading light) |
| HRP conjugate Diluent for IL1β | 10 mL 1 vial | 4ºc |
| (25 ×) Concentrated Wash Buffer for IL1β | 30 mL 1 vial | 4ºc |
| IL1β Substrate Reagent | 10 mL 1 vial | 4ºc (shading light) |
| Stope solutions for IL1β | 10 mL 1 vial | 4ºc |
| Plate sealers | pieces about 5 | |

## 3-4 Methods

### 3-4-1 Media and biological material

### 3-4-1-1 : Preparation of culture media

The culture media were prepared in accordance with the manufacturer's instructions and autoclaved for 1212 and 15 minutes to sterilize them (Macfadden, 2000).

### 3-4-1-1-1-: Muller-Hinton agar

The manufacturing company received ready-to-use Muller-Hinton agar medium for use in antimicrobial susceptibility testing (Forbes et al., 2007).

**3-4-1-1-2-: MacConkey agar medium**

It was utilized to separate lactose fermenters from non-lactose fermenters and for the first separation of the majority of Gram-negative bacteria (Winn et al., 2006).

**3-4-1-1-3 : Brain heart infusion broth**

It was employed to activate bacterial isolates (Forbes et al.,2007).

**3-4-1-1-4-: Blood agar medium**

Following the manufacturer's directions, 1000 cc of D.W. was used to dissolve 40 g of blood agar base. to make a medium with blood agar. Fresh human blood was added in 5% after the medium was cooled to 50 oC. after it had been autoclaved at 121 oC for 15 minutes at 15 psi of pressure. This medium was used to cultivate the bacterial isolates and test their ability to hemolyze blood as an enrichment media. (Forbes *et al*., 2007)

**3-4-1-1-5 - : Peptone water medium**

To create this medium, 8 g of peptone were dissolved in 1000 cc of distilled water., autoclaving it at 121 oC for 15 minutes at 15 psi, and then dividing it among test tubes. It was utilized to demonstrate how bacteria may break down the amino acid tryptophan to produce indole. (MacFaddin, 2000).

**3-4-1-1-6 -: Methyl red – vogas-proskauer medium (MR-VP)**

To determine if glucose had partially or completely hydrolyzed, MR-VP medium was produced. (MacFaddin, 2000).

**3-4-1-1-7-: 5% glycerol in a brain-heart infusion broth**

To create this medium, 5 ml of glycerol were combined with 95 ml of BHI broth, and it was then autoclaved. for 15 minutes at 121 oC and 15 psi of pressure (psi). The medium was employed to preserve bacteria. (MacFadden, 2000).

**3-4-1-1-8-: Simmon's citrate medium**

Simmon's citrate medium was used to test if bacteria could only use citrate as a carbon source. (MacFaddin, 2000)**.**

**3-4-1-1-9- : Kligler iron agar**

As a first step in the identification of Gram negative bacilli, glucose and lactose fermentation as well as potential hydrogen sulfide (H2S) production were determined using Kligler iron agar. (McFaddin, 2000).

**3-4-1-1-10-: Mannitol salt agar**

This medium was made in accordance with the manufacturer's instructions. It was employed as a selective medium for the differentiation and isolation of Staphylococcus species and contains 7.5–10 naphthalene, which is selective for Staphylococcus and Micrococcus and differential for Staphylococcus. (Macfaddin, 2000).

**3- 4- 2: Solutions and reagents**

### 3- 4- 2-1- : Catalase reagent

The substance was made by mixing 100 ml of distilled water with 3% H2O2, and then kept in a shadowy bottle. The reagent was used to identify bacteria's capacity in order to catalyze the enzyme. (Forbes *et al*., 2007).

### 3- 4- 2-2-: Oxidase reagent

Tetra methyl—paraphenylene diamine di hydrochloride, 0.1 g, created the reagent by dissolving in A dark bottle was used to store 10 ml of distilled water. The substance served to identify the ability of bacteria to produce the Oxidase enzyme, and it was newly synthesized. (Forbes *et al*., 2007).

### 3- 4- 2-3- : reagents of Methyl red

To make the solution, 300 ml of 99% ethanol and 0.1g of methyl red were combined. solution. reagent. adding distilled water to bring the amount to 500 ml. This reagent was used to confirm the full hydrolysis of the glucose. (MacFaddin, 2000).

### 3- 4- 2- 4-: Barrett's reagent

According to the procedure described by (Winn et al., 2006), the reagent was made as follows:-

Dissolved 100 ml of 99% ethanol alcohol was used to dissolve 1 to 5 grams of naphthol, which was then stored in a dark bottle in a cool location. In 100 ml of distilled water, 2-40 g of KOH were dissolved. This test enhances the partial fermentation that results in the creation of butylene glycol because it was utilized to identify acetone formation in the culture media.

### 3- 4- 2- 5- : Kovac´s reagent

25 ml of concentrated HCl was added after 5g of (P-dimethyl aminebenzaldehyde) had been dissolved in 75 ml of amyl alcohol. the substance used to identify indole (MacFaddin, 2000).

### 3- 4- 2- 6- : Gram stain solution

The Syrbio firm supplied the Gram Stain solution. Bacterial cells that are Gram positive and Gram negative, as well as their shape and organization, were studied using the solution. (Forbes *et al.*, 2007).

### 3- 4-2-7- Tris borate EDTA (TBE) buffer (bio-basic / England )

500 ml of distilled water (d H2 O) were combined with 50 ml of TBE (10X) stock solution to produce 500 ml of TBE (1X). To a final volume of 500 ml of d H2 O, 50 ml of TBE (10X) stock solution and 500 ml of TBE were added (0.5X)was created.

### 3- 4- 2- 8- Loading dye

During DNA electrophoresis, the DNA band was trafficked using this loading dye.

Ethidium bromide stain, 3-4-2-9

For agarose and page gel electrophoresis, it is an intercalating substance frequently used as a fluorescent tag (nucleic acid stain).

## 3-5 : Methods

### 3-5-1: - study population

200 samples from breast cancer patients were used for cultivation, including 70 biopsies, 100 blood and serum samples, 30 blood and serum from patients who had undergone chemotherapy, and 50 control apparently healthy involved (blood and serum) from healthy women. The tissue was taken 5 cm outside the marginal zone. Fresh tissue was immediately put in a sterile plane tube or pee cup following excision. It has a typical saline solution within. TLR2, TLR4, IL1, and IL1 polymorphism were performed on biopsies. 3 samples were performed to assess the mucosal immune markers CA 15-3, TLR2, IL1, and IL1. In order to measure immune markers systemic (CA 15-3, TLR2, IL1, IL1) with age ranging from (14-66) years, serum separated from blood samples using gel tubes were collected from patients with breast tumors and healthy women during the months of September 2021 and October 2022 at AL-Hilla-Teaching Hospital, Imam Sadiq Hospital, and Marjan Hospital.

### 3-5-2- Data collection and questionnaire

All participants in this study were given a questionnaire to fill out in order to learn more about their 1-Related history of breast tumors.

two-family breast cancer history

3-Age of the relative at the time of her breast cancer

4-Cancer date

5 different breast diseases

6 Relationship status

### 3-5-5 blood specimen

Each patient provided five milliliters of venous blood, of which one milliliter was placed in EDTA tubes and the other four milliliters were slowly pumped into disposable tubes containing separating gel. While blood in gel-containing tubes was allowed to coagulate at ambient temperature for 30 minutes and then centrifuged at 2000 x g for around 15 minutes, the serum was collected and stored at -20oc until utilized, blood in EDTA tubes was stored at -20oc to be used later in genetic research..(NCCLS, 2003) .

### 3-5-6 -: Gram positive and negative bacterial isolation and identification

Each positive and negative A single colony was produced by the culture, and it was then recognized using its morphological characteristics (colony size, form, pigment color, and kind), translucency, edge, elevation, and texture).To examine a particular form, reaction type, aggregation, and particular intracellular chemicals, colonies were then stained using the gram stain method. (2006) Winn et al.

### 3-5-7 Diagnostic tests

when the incubation phase was ended. Colonies with diverse characteristics were put through a variety of tests, and they were identified using (Macfaddin ,2000 ; Forbes *et al*.,2007).

### 3-5-7-1 Microscopic examination

Gram stain was used to determine whether a small portion of earlier colonies were Gram positive or Gram negative and to reveal the type of bacteria present.

### 3-5-7-2 Biochemical tests

#### 3-5-7-2-1-: Catalase test

An enzyme called catalase helps hydrogen peroxide release its oxygen. After a A A small amount of bacterial growth was applied with a clean, dry glass slide and a sterile wooden stick. After that, 3% H2O2 was dropped in. bubbles of gas were seen, indicating a successful outcome. (Collee *et al.*, 1996).

#### 3-5-7-2-2-: Oxidase test

Tetramethyl-phenylenediamine dihydrochloride, a reverse Specific bacterial oxidases were necessary for this test in order to facilitate the transfer of electrons between the bacteria's electron donors and the dye, which was then turned a deep purple color.. Using a wooden stick, a few of the bacterial colonies were scattered throughout the filter. paper. after it had

been saturated with freshly made oxidase reagent in a petri dish. A successful outcome was indicated by the smear's color changing from pink to purple in less than 10 seconds (Forbes *et al*., 2007).

### 3-5-7-2-3-Coagulase test

This test was used to determine whether the bacteria being examined could make coagulase, an enzyme-like protein that clots plasma that has been oxidized or citrated. The test was carried out in the following manner: A 1:15 dilution of citrated human plasma was combined with an equal volume of an overnight bacterial broth culture, all of which were then incubated at 37°C. The test is successful if clots form in 1 to 4 hours. As a control, a tube of plasma diluted in sterile broth was used. (Brooks *et al*., 2007).

### 3-5-7-2-4-: Indole test

For this assay, bacterial growth was introduced into the peptone water medium via the loop, which was then cultured for 24-48 hours at 37 oC. Kovac's reagent was added in 6–8 drops for the drug test (p-dimethyl amino benzaldehyde in amyl alcohol). the appearance of a ring with a red color at the top of the soup was indicative of a favorable reaction. (MacFaddin, 2000).

### 3-5-7-2-5-: Methyl –red test

Select The MR-VP broth tubes were loaded with bacterial colonies, and they were subsequently incubated for 24 to 48 hours. at 37 oC. Then, The methyl red reagent was added in five drops. Red's appearance and observation denote a productive outcome and complete glucose hydrolysis. (MacFaddin, 2000).

### 3-5-7-2-6-: Vogues –proskauer test

The MR-VP broth tubes were filled with chosen bacterial colonies, and they were subsequently incubated for 24 hours at 37 oC. Before reading, reagent A should be added together with 0.2 ml of 40% KOH solutions and 0.6 ml of alpha nephthol. The presence of red hue indicates a successful outcome. after 15 minutes because glucose partially hydrolyzes to produce acetone or acetyl methyl carbinol (MacFaddin, 2000).

### 3-5-7-2-7-: Citrate utilization test

Before the bacterial colonies were added, Simmon's citrate medium was autoclave sterilized and cultured for 24 hours at 37 degrees Celsius.Positive results were indicated by a shift in the medium's color from green to blue with growth streaks, while negative results were indicated by a return to the natural color of green without growth. (Winn *et al*., 2006).

### 3-5-7-2-8 -:TBE Buffer (Tris-Borate-EDTA)

The most used buffer for DNA and RNA gel electrophoresis was TBE running buffer. TBE was made and kept in stock as a 10 solution. In 1000 ml of D.W., 108 g of Tris base, 55 g of boric acid, and 40 ml of 0.5 M EDTA were dissolved to create the 10 working solution. However, 900 ml of sterile D.W. and 100 ml of 10 TBE buffer were combined to create the final concentration of 1 TBE solution. (Sambrook and Russel, 2001).

**3-5-7-3 -: Vitec 2 system**

The biochemical test was verified using the Vitec 2 system in accordance with the manufacturer's recommendations. This system consists of a personal computer, a reader incubator, as well as numerous internal components like a cassette loading mechanism, a card sealer, a card cassette, a bar code reader, and an incubator. Instruments control electronics, waste processing, and firmware in addition to transmittance optics. In order to improve the effectiveness of microbiological diagnosis and decrease the need for additional tests, the system was outfitted with an enlarged identification data base. This will increase the safety of the test as well as the users.

The next several steps are all set up in accordance with the manufacturer's instructions. A loop containing a single colony is injected into a test tube with three ml of normal saline. The colony needs to be 24 hours old. A dens check machine was used to standardize the colony to 1.5 108 cells per

milliliter; McFarland's standard solution. A barcode was used to insert a sample identification number into the computer software once the standard inoculums had been loaded onto the cassette. The sample ID number and VITEK 2 card were consequently matched. The cassette was moved from the reader incubator module to the filler module. once the cards had been filled. The apparatus maintains the temperature of the incubation chamber, reads the cards optically, continuously monitors the test results, and sends the data to a computer for analysis.

**1-Standardization**

Simple inoculum preparation, standardization, and dilution steps are all that is required to minimize handling after primary isolation. After the standard inoculum was introduced to the cassette, a sample identification number was entered into the computer software using a barcode.

**2-Traceability**

The barcode applied to the card during manufacturing is then scanned to determine the VITEK 2 card type, and the card is attached to the sample ID. In one simple barcode reading process, manufacturer barcodes connect the card to patient data.

**3-Load and Go**

Activate the filler mode and insert the tape. Transfer the cassette to the reader/incubator module once the cards have been loaded. The instrument manages all future actions.

**3-6 Blood specimen**

**3-6-1 Molecular analysis : Genomic DNA mini kit (blood).**

Using the Favorgen kit (FABG 100 preps), human genomic DNA was isolated from frozen white blood cells (WBC) in order to detect and amplify the IL-1 511, IL-1-31, IL1 -889 C>T, TLR4 +3725G/C, TLR4 Thr399Ile, and TLR2 Asp299Gly genes..

### 3-6-1-1- Extraction of DNA steps from frozen blood for molecular study

The following procedures were used to harvest human genomic DNA from healthy control subjects and those with periodontitis:

1- 200 l of blood were transferred to an unsupplied 1.5 ml micro centrifuge tube.

Add the required amount of PBS if the sample volume is under 200 l.

2- 30 l of proteinase K (10 mg/ml, not provided) should be added to the sample, and it should be stirred for a short while. then incubate for 15 minutes at 60 °C.

3. The specimen was added to 200 l of FABG buffer, which was then mixed by vortexing.

4-Lyse the sample by incubating it for 15 minutes in a 70 °C water bath. Every three minutes during incubation, flip the sample over.

Elution Buffer, number five, For DNA elution, a 70 oC water bath must be preheated.

If RNA-free demonic DNA is required, add 5 ml of 10 mg/ml RNase A to the sample and vortex to combine.

At room temperature, incubate for five minutes.

The material was added to 8-250 l of ethanol (96–100%) and vortexed for 10 seconds. Using a pipette, thoroughly mix the sample if any precipitate has developed.

9FABG Column was utilized. Place in a collection tube . Transfer the sampleS mixture with care to the FABG Column .

1 minute of centrifuging at 14,000 rpm or 18,000 x g speed. Place the FABG Column in a new Collection Tube and throw away the old one.

W1 Buffer in the amounts of 10-400 l was added to the FABG Column, and the centrifuge was run for 30 seconds at 14,000 rpm or 18,000 x g. Place the FABG Column back into the Collection Tube after discarding the flow-through.

Wash Buffer in the amount of 11–600 l was added to the FABG Column , and the centrifuge was run for 30 seconds at 14,000 rpm or 18,000 x g.

Place the FABG Column back into the Collection Tube after discarding the flow-through.

—

When opening the Wash Buffer for the first time, make sure ethanol has been poured.

12- To dry the column, centrifuge for an additional three minutes at a speed of 14,000 rpm or 18,000 x g.

—Critical Step The next enzymatic reactions won't be hampered by leftover liquid thanks to this step.

13- A fresh 1.5 ml micro centrifuge tube was filled with the dried FABG Column.

The membrane core of the FABG Column was added with 14-100 l of preheated elution buffer or TE.

15- The FAGB Column was incubated in an incubator for 10 minutes at 37 oC.

16- To elute the DNA, centrifuge for one minute at full power (18,000 x g or 14,000 rpm) —

100 l is the standard volume for elution. Repeat the DNA elution process to maximize DNA recovery if a larger DNA yield is desired; the final volume may be 200 l.

17- The DNA fragment was kept at 4 or -20 degrees Celsius.

**3-6-1-2- Extraction of DNA steps from tissue for molecular study**

The intended organ was Remove from a human

Second, the prepared sample was Cut off using a scalpel or scissors to the proper size.

3 The sample material was thinly cut Place the sample into a grinding jar (mortar), add liquid nitrogen, maintain the sample covered in the liquid nitrogen, and gently disturb until the sample is entirely homogenized. Then, allow the liquid nitrogen to evaporate before moving on to step 4.

4- Using a spatula, transfer 25 mg of the powdered tissue sample into a 1.5 ml tube.

200 ml of buffer CL, 20 ml of proteinase K, and 5 ml of RNase in 5 A solution was added to a sample tube and aggressively vortexed to combine it.

6- The lysate was incubated at 56°C for 10–30 minutes using a heated heat block or water bath.

7- 200 l of buffer BL should be added to the top sample tube and properly mixed after the lysis is complete. then wait five minutes at 70°C while the mixture sits.

8- To eliminate unlysed tissue fragments, the sample tube was centrifuged at 5 minutes at 13000 rpm. In a subsequent step, cautiously transfer 350–400 l of the supernatant into a brand-new 1.5 ml tube.

9. To collect the drops from the lid, quickly centrifuge the 1.5 ml tube.

Using a pulse vortex, absolute ethanol (10–200 ml) was added to the lysate and thoroughly mixed. After a brief mixing, centrifuge the 1.5 ml tube to get rid of any drips from the lid.

11- Carefully applying the liquid to the spin column (in a 2 ml collection tube) without wetting the rim, capping the container, and centrifuging at 13000 rpm for one minute were the procedures used. Place the spin column in a fresh 2 ml collection tube after discarding the filtrate.

12- 700 l of buffer WA were added to the spin column without wetting the rim, and the centrifuge was run at 13000 rpm for one minute. Reuse the collection and throw away the flow-through

The spin column was filled with 13-700 l of buffer WB, without the rim, and centrifuged for one minute at 13000 rpm. Place the column into a new 2.0 ml collection tube and discard the flow-through. Next, centrifuge the tube once more for an additional minute to dry the membrane.

14- A new 1.5 ml tube was used to hold the spin column, and 30-100 l of buffer CE was added directly to the membrane. The spin column was then incubated for 1 minute at ambient temperature before being centrifuged for 1 minute at 13000 rpm to elute.

**Diluting of primers**

The primers used in this work were from Bioneer, and they were made in a clean room following strict ISO 9001:2000 guidelines to provide a DNase/RNase and DNA-free environment.

Primer were frequently sent in a lymphilized state. The lymphilized primer units were distributed in pico mole sizes as they accumulated. the quantity of sterile DW that was added to each primer to create the stock that will eventually be utilized in PCR.

According to the oligos manufacturer, the primers were diluted as follows: The primer tubes were spun down before the primer caps were opened.

2-To acquire a size of 100 pmoles /, the necessary amount of water was applied to each primer.

3 correctly vortex the primers back into suspension.

To create diluted primer for PCR amplification, 4-A10 l of primer stock was transferred to a 1.5 ml epindorff tube that contains 90 l of sterile DW.

5 The priming stock was kept at -20 degrees.

### 3-7 PCR amplification of human IL1α, IL1β,TLR2 and TLR4

The components of the Bioneer master premix, which was employed in the PCR amplification, are mentioned in the table ( 3-10 )

**Table (3-10) Master mix components that used in PCR amplification**

| Item | Concentration |
|---|---|
| Tag DNA polymerase | 1U in 1 µl |
| dTTP , dGTP , dCTP and dATP | 250 mM |
| Tris –HCl (pH 9.0) | 10 mM |
| KCl | 30 mM |
| $MgCl_2$ | 1.5 mM |
| Stabilizer and tracking dye | 5 mM |

### 3-7-1 Amplification of IL-1β and IL-1α

#### 3-7-1-1 Interleukin 1 beta (IL-1β )

The primers of Interleukin beta are listed in the table below :

**Table ( 3-11) Interleukin 1 beta (IL-1β ) primers and references**

| Primers | Sequence | Amplicon | References |
|---|---|---|---|
| IL-1β-31 | | | |
| Forward | 5′-AGA AGC TTC CAC CAA TAC T-3′ | **240** | Akisikand Dalay , 2007 |
| Reverse | 5′-TAG CAC CTA GTT GTA AGG A-3′ | | |

#### 3-7-1-1-3 Interleukin 1 beta (IL1β ) 31T

Annealing temperature and PCR conditions were given in Akisikand and Dalay (2007). Amplified DNA fragments were placed in 3.0% agarose, TBE buffer concentration was 0.5x, electrophoresis period was 60 minutes at 80 volts, and the bands of IL-1ß were seen under a UV light illuminator after

ethidium bromide had been added. Using a 100 base-pair ladder as a DNA marker, fragment sizes and PCR conditions were estimated. was listed in the table ( 3-11) .

**Table ( 3-11 ) PCR condition of Interleukin 1 beta (IL1β ) C31 in breast tumor study**

| Stage | Steps | Temp. (C°) | Time (min) | Cycle |
|---|---|---|---|---|
| 1 | Initial Denaturational | 94 | 5 | 1 |
| 2 | DNA denaturation | 94 | 1 | 25 |
| | Primer annealing | 54.8 | 1 | |
| | Extension | 72 | 1 | |
| 3 | Final extension | 72 | 5 | 1 |
| 4 | Hold | 4 | | |

## 3-2-7-4 -2 AluI restriction enzyme ( thermo ) :

A single nucleotide polymorphism in the promoter region of IL - 1B is caused by changing the nucleotide at position -511 there. Using a particular primer, a recognition site for the restriction enzyme AluI was found. However, the AluI restriction enzyme utilized in this work was used for this SNP, and the enzyme recognized the sequence :

5 ' … A G↓C T… 3

3 ' … T C↑G A…5 ' .

Sources of AluI : *Arthrobacter luteus*

## 3-2-7-4 -3 -: The components and reaction protocol

AluI enzyme digestion was performed in a volume of 60µl The following protocol was considered an example of a typical restriction enzyme digestion .

1. In a sterile tube , the following components were assembled according to thermo company table ( 3-12)

**Table (3-12) component of REFLP –PCR**

| Components | Volume µl |
|---|---|
| PCR reactions mixture | 10 |
| Nuclease free water | 18 |
| 10X buffer tango | 2 |
| Restriction Enzyme *AluI* 600u/ µl | 1 |
| Final volume | 31 |

2. All components were mixed gently by pipetting , and the tube was closed without using vortex .

3. All components with restriction enzyme were incubated at the 37C ° for 1-16 hour .

4. Electrophoresis in polyacrylamide gel was done.

**3-7-1-2 Interleukin 1 alpha IL1 α -889 C>T**

The annealing temperature and PCR conditions were disclosed in (Emiroullar *et al*., 2018). After adding ethidium bromide and running the electrophoresis for 60 minutes at 80 volts with the amplified DNA fragments in 3.0% agarose, the bands of IL-1ß were visible under a UV light illuminator. A 100 base-pair ladder was used as a DNA marker for calculating fragment sizes; Table 3-13 lists the PCR parameters.

**Table ( 3-13 ) PCR condition of IL1 α -889 C>T in breast tumor study**

| Stage | Steps | Temp. (Cº) | Time (min) | Cycles |
|---|---|---|---|---|
| 1 | Initial Denaturation | 94 | 5 | 1 |
| 2 | DNA denaturation | 94 | 1 | 35 |
| | Primer annealing | 53.9 | 1 | |
| | Extension | 72 | 2 | |
| 3 | Final extensional | 72 | 5 | 1 |
| 4 | Hold | 4 | | |

## 3-2-7-5 -1 Nco I restriction enzyme ( thermo ) :

IL1 α -889 C>T has a single nucleotide polymorphism that is caused by changing the nucleotide at position -889 C>T in the promoter region. Utilizing a particular primer, a recognition site for the restriction enzyme Nco I was found. However, the restriction enzyme Nco I utilized for this SNP in this investigation was able to detect the sequence :

5 ' … C↓C A T G G … 3

↑

3 ' … G G T A C C…5 ' .

Source of AluI : *Arthrobacter luteus*

## 3-2-7-5 -2 -: The components and reaction protocol

Nco I enzyme digestion was performed in a volume of 50µl The following protocol was considered an example of a typical restriction enzyme digestion .

1. In a sterile tube , the following components were assembled according to thermo company table ( 3-14).

**Table (3-14) component of REFLP –PCR**

| Components | Volume µl |
|---|---|
| PCR reaction mixtures | 10 |
| Nuclease free water | 18 |
| 10X buffer tango | 2 |
| Restriction Enzyme *Nco I* 500u/ µl | 1 |
| Final volumes | 31 |

2. All components were mixed gently by pipetting , and the tube was closed without using vortex .

3. All components with restriction enzyme were incubated at the 37C ° for 1-16 hour .

4. Electrophoresis in polyacrylamide gel was done

### 3-7-2 Toll like receptor 2

The annealing temperature and PCR conditions were described in (Theodoropoulos et al., 2012). Amplified DNA fragments were placed in 2.5% agarose, 0.5x TBE buffer concentration, 60 minutes at 80 volts, and TLR2 bands were seen under a UV light illuminator. For the determination of fragment sizes, a 100 base-pair ladder was employed as a DNA marker; the PCR conditions are indicated in the table. ( 3-15) .

**Table ( 3-15 ) PCR condition of TLR2 in breast tumor study**

| Stage | Steps | Temp. (Cº) | Time (min) | Cycles |
|---|---|---|---|---|
| 1 | Initial Denaturation | 95 | 5 | 1 |
| 2 | DNA denaturation | 95 | 30S | 35 |
| | Primer annealing | 62 | 40S | |
| | Extension | 72 | 40S | |
| 3 | Final extension | 72 | 7 | 1 |
| 4 | Hold | 4 | | |

### 3-7-4 -1TLR4 +3725G/C SNP

(Zamzam et al., 2019) presented the PCR and annealing temperature conditions. Amplified DNA fragments were placed in 2.5% agarose, TBE

buffer was added at a concentration of 0.5x, and the bands from George et al., 2012 were observed using a UV light illuminator. For the determination of fragment sizes, a 100 base-pair ladder was employed as a DNA marker; the PCR conditions are indicated in the table. ( 3-16) .

**Table ( 3-16 ) PCR condition of 1TLR4 +3725G/C in breast tumor study**

| Stage | Steps | Temp. (Cº) | Time (min) | Cycles |
|---|---|---|---|---|
| 1 | Initial Denaturation | 95 | 6 | 1 |
| 2 | DNA denaturation | 94 | 1 | 35 |
| | Primer annealing | 57.5 | 1 | |
| | Extension | 72 | 2 | |
| 3 | Final extension | 72 | 10 | 1 |
| 4 | Hold | 4 | | |

## 3-2-7-7-1 Ear I restriction enzyme ( thermo ) :

The nucleotide at nucleotide position +3725G/C of TLR4 contains a single nucleotide polymorphism as a result of the change. Using a particular primer, a recognition site for the restriction enzyme Ear I was found. However, the restriction enzyme Ear I utilized in this investigation to detect this SNP was able to recognize the sequences :

5 ' … C T C T T C (N) ↓. 3

3 ' … G A G A A G (N)↓..5 ' .

Sources of Ear I : *E. coli*

### 3-2-7-7-2 -: The components and reaction protocol

Ear I enzyme digestion was performed in a volume of 30µl The following protocol was considered an example of a typical restriction enzyme digestion .

1. In a steriles tubes , the following components were assembled according to thermo company table ( 3-17)

**Table (3-17) component of REFLP –PCR**

| Components | Volume µl |
|---|---|
| PCR reaction mixture | 10 |
| Nuclease free water | 18 |
| 10X buffer tango | 2 |
| Restriction Enzyme Ear I 300u/ µl | 1 |
| Final volume | 31 |

2. All components were mixed gently by pipetting , and the tube was closed without using vortex .

3. All components with restriction enzyme were incubated at the 37C ° for 1-16 hour .

4. Electrophoresis in polyacrylamide gel was done

**3-8 Step of gel electrophoreses on agarose for IL1α , IL1β , TLR2 and TLR4 (Lewis , 2011) .**

1-A plastic tray was used to hold the gel-casting tray, and the comb's teeth were positioned about 0.5mm above the gel's bottom.

2. 50 ml of TBE (10X) stock solution was combined with 500 ml of deionized water to make 500 ml of TBE (1X).

3. Added 0.8g of agarose and 100ml of the buffer to a 500ml flask. By placing the solution on a hot plate for about 10 minutes, you can melt the agarose. Make sure the agarose is dissolved, or that no agarose particles are visible, by carefully swirling the agarose solution.

4- Red safe stock solution was added after the agarose solution had been cooled. The agarose was carefully poured into the gel-casting tray, with any air bubbles being eliminated with a yellow tip.

5- About 1.5 cm from the edge of the gel was where the comb was placed. Agarose was permitted to set up for roughly 20 to 30 minutes. A gentle back and forth motion was taken to remove the comb once the agarose had solidified.

Sixth, the gel-casting tray was removed and set on the gel box's central supporting platform.

7- The buffer chamber was topped off with electrophoresis buffer so that it was 0.5–1 cm above the gel's surface.

8- Using a point, specimens were placed into the wells. Just above the well, the tip was positioned beneath the electrophoresis buffer's surface. The specimen was released gradually so that it might hit the well's bottom. 9-

Next, 5 ul of DNA material was loaded in the other well after a Sul of DNA molecular weight marker.

10- When DNA had moved toward the positive (red) electrode positive away from the well, the lid was put on the gel box and the electrodes were linked. After the tracking dye had traveled at least 10 cm along the gel's length, the power was turned off.

## 3-9 Step of gel electrophoreses on PAGE for IL1α , IL1β , TLR2 and TLR4

### 3-9-1 Preparation of PAGE gel and steps od protocol

This procedure was followed as stated in (Brown , 2000).

1-A 100 ml of DW was used to dissolve 29 g of acrylamide and 1 g of bis-acrylamide. The mixture was stirred until the solution was clear.

2. The solution was held at 4oC after being filtered using 45mm filter paper.

3-The apparatus for vertical electrophorases was ready 4-The elements listed in table (3-14) were mixed

**Table (3-14 ) component of PAGE electrophorases**

| Components | Volume |
|---|---|
| 30% acrylamide/bis (29:1) | 8ml |
| 10x TBE | 1 ml |
| TEMED | 40µl |
| 10% ammonium persulfate | 400µl |
| Distill water | 20.8ml |

**5-The mixture was poured into the machine.**

**6-The comb was inserted into the gel and left there for 30–40 minutes until the gel polymerized before being withdrawn.**

**The apparatus's lid was then put on after adding 0.5 to 1 times the recommended amount of TBE buffer to the tank and loading 7-A20 l of amplified DNA into the wells.**

**8 - Activate the power source for three hours.**

### 3-10 Photo documentation

Agarose gel was viewed using a UV trans illuminator that came with the gel documentation device. Agarose was placed over the UV trans illuminator's tray, and UV light was then made visible. A digital camera from Canon was used to capture the outcomes.

### 3-11 Estimation of IL1α , IL1β , TLR2 and TLR4 Concentration in tissue and serum by ELISA test

#### 3-11-1 Fundamentals of assay

This ELISA kit employs the sandwich-ELISA methodology. The human IL-1alpha, human IL-beta, and human TLR2 antibodies have been pre-coated on the micro ELISA plate included in this kit (three kits were used separately, but the same data has been given). Stands or samples are placed in the appropriate micro ELISA plate wells, which are then combined with the chosen antibody. Then, each microplate well was finished. is then incubated with a human-specific biotinylated detection antibody. IL-1alpha,

IL-1 beta, and TLR2 before being added to and treated with an Avidin-Horseradish peroxidase (HRP) conjugate. By washing, free components are taken out. The substrate solution is added to each well. IL-1alpha, IL-beta, and TLR2 exclusively found in human wells The color of the Avidin-HRP combination and the biotinylated detecting antibody is blue. The addition of stop solution stops the enzyme substrate action, which results in the color turning yellow. A wavelength of 450 nm plus 2 nm is used to spectrophotometrically determine the optical density (OD). Human IL-1alpha levels and the OD value have a linear relationship. You can determine the amount of human DNA by comparing the OD of the samples to the standard curve. IL-1alpha, IL-1beta, and TLR2 in the sample.

**3-11-2-: Reagent preparation**

Before use, all reagents were brought to room temperature (18–25 c).

Human IL1-, IL1-, and TLR2 kit components and preparation procedures include:

**A- Wash buffer :**

750 mL of wash buffer were created by diluting 30 mL of concentrated wash buffer with deionized or distilled water. Remaining solution was refrigerated at 4 ° C. If crystals have developed in the concentrate, a water bath heated to 40 °C was used to gently mix the crystals until they were entirely dissolved. The solution was chilled to room temperature before use.

**B- Standard :**

Standard was made 15 minutes prior to use. Standard was reconstituted with 1.0 ml of reference standard and specimen diluent after being centrifuged at 14000 rpm for 1 minute. After tightening the lid, spin the

container several times while letting it stand for 10 minutes. It was properly blended with a pipette once it had entirely dissolved. This reconstitution was made from a 500 pg/ml stock solution, and any necessary serial dilutions were made after that. The following concentrations were advised: 500 , 250 , 125 , 62.5 , 31.25 15.625 , 7.813 , 0 pg / ml A 0.5ml standard at 500pg/ml was taken and added to an eppendorf tube together with 0.5ml of reference standard and specimen diluent to create a standard solution with a concentration of 250 pg/ml. The preparation methods for the remaining concentrations, however, were all the same. The undiluted standard is the highest standard (500pg/ml). The reference standard and sample diluent served as the zero (0 pg/ml).

**C- Biotinylated detection Ab**

Prior to the experiment, the necessary amount was determined (100 l/well). In practice, an additional 100-200 l were prepared. The concentrated biotinylated detection Ab was diluted to the working concentration using biotinylated detection Ab diluent (1: 100) after centrifuging the stock tube before use.

**D- Concentrated HRP conjugate**

Prior to the experiment, the necessary amount was determined (100 l/well). In practice, an additional 100-200 l were prepared. The concentrated HRP conjugate was diluted to the working concentration using concentrated HRP conjugate diluent (1: 100).

**E- Substrate reagent**

The vial of this reagent doesn't open until it is required since it is sensitive to impurities and light. With sanitized tips, the required amount of the

reagent was sucked, and any remaining, unneeded amount was poured back into the vial.

**3-11-3 Procedure of ELISA test for tissue and serum of IL 1α, IL1β and TLR2**

**1. Specimen addition : •**

100 l of standard, blank, or specimen solution were given to each well. The well-liked blank reference standard and sample diluent. Solutions were gently mixed and put to the bottom of the micro ELISA plate without contacting the walls. The plate was then covered with sealer and incubated at 37 ° C for 90 minutes.

**2. Biotinylated detection Ab addition : .**

Without washing, the liquid was drained from each well. Working solution containing 100 l of biotinylated detection Ab was added to each well right away. In order to achieve full mixing, the plate was covered with sealer and gently moved. It was then incubated at 37 ° C for one hour.

**3. First wash step :**

Three times each well was aspirated, washed, and the procedure was carried out.

Each well was washed by being completely drained of liquid at every step (around 350 liters of wash buffer per well). Remaining wash buffer was removed by aspirating or decanting after the final wash.

Inverted onto a piece of thick, clean, absorbent paper, the plate was then placed.

**4 . HRP Conjugate addition : .**

Each well received 100 l of the HRP conjugate working solution.

The plate was sealed and incubated for 30 minutes at 37 ° C.

**5. Second wash step :**

The washing procedure was carried out as described in step 5 five times ( 3)

**6. Substrate addition :** .

To each well, 90 l of substrate solution were added.

A fresh sealer was applied to the plate. and 15 minutes of incubation at 37 ° C. Light was kept off the plate.

According to the actual color shift, the reaction time was either cut or prolonged, but not by more than 30 mints.

the reaction was stopped when an apparent gradient color appeared in the standard wells.

**7. Stop of reaction :**

• To each well, 50 l of stop solution were added. The tint then abruptly changed to yellow.

**8. OD Measurement of specimens** :

• A micro-plate reader set to 450 nm was used to determine the optical density (OD) of each well at once.

9. After experiment ended , all the unused reagents was put back into the refrigerator according to the specified storage temperature .

**3-12 Calculating of results of ELISA test**

When specimens were diluted, the concentration calculated from the standard curve was multiplied by the dilution factor. The mean OD value for each standard was graphed on the y-axis versus the concentration on the x-axis to create the normal distribution.. After the proper dilution, the

specimen's OD that had exceeded the upper limit of the standard curve was retested.

### 3-13 Statistical Analysis

The SPSS 19 version was used for all statistical analysis. (Mean SD) was used to express the data. The concentration of ELISA for IL1, IL1, and TLR2 was estimated using the trend tool in Microsoft Excel 2010. T independent test was used to determine whether the distribution of all variables was normally distributed. The Chi-square () test was used to analyze the differences between the healthy control participants and the frequency of periodontitis patients. Using the Hardy Weinberg equilibrium, the genetic polymorphism of the IL-1, IL-1, TLR-2, and TLR-4 genes was done. The risk factor was estimated using the odds ratio (OR). P values under 0.05 are regarded as significant.

## 4-1 Characterization of breast tumors disease

The women with breast tumors divided according to the types of breast tumors age, history of disease , in table ( 4-1) The percentage and number who in age (14-29) years was 48 (28%) , in age (30-45) years was87(51%) and in age (46-66) was 35(21%) this study show the most common infection in woman with breast tumors in age group (30-45)

### Table (4-1) Demographic of subject

| **Patients (170 women)** | | | **Control (50 women)** |
|---|---|---|---|
| Number ( percentage %) | | | |
| Age years | ( 14-29) | 48 (28%) | 18(36%) |
| | (30-45) | 87(51%) | 27(54%) |
| | (46-66) | 35(21%) | 5(10%) |
| Family history | Present | 50(29%) | |
| | Absent | 120(71%) | |
| Status | Married | 104(61%) | |
| | Unmarried | 66(39%) | |
| Types of feeding | Breast feeding | 51(32%) | |
| | Non- breast feeding | 44(27%) | |
| | Mix feeding | 66(41%) | |
| Types of breast tumors | Benign breast tumors | 96(56%) | |
| | Malignant breast tumor | 74 (445%) | |
| Types of Benign breast tumors | Fibrocystic change | 27(28%) | |
| | Fibro adenoma | 49(51%) | |
| | Granulomastitis | 20(21%) | |

This study was disagree with study (Alwan, 2010) also these study was disagree with study (Majid *et al*., 2017)

Age was a risk factor for breast cancer evolution , the may be due to increased chromosomal damages as a result of repeated divided in age increasing, which lead to the accumulation of mutations in the DNA that bring about to cancer development and "the age-related increase in chromosomal harm occurred hurry in women than in men" because the increasing level of aberrations, and rise in the level of X chromosome damage was the main contributor of aging in women (Wojda *et al*., 2006 and Orta and Günebakan, 2012). But this study was agree with study (Uyisenga *et al*.,2020 )

This study was agree with study (Al-Rawi, 2013 ) in Erbil Iraqi showed that.

But this study showed the most common types of breast tumors was benign than malignant this agree with study (Alwan , 2010) in Iraqi who showed that number and percentage of but this study was disagree with study (Al-Rawi, 2013) show that 36 cases of malignant breast lesions fond in studied patients. Most of patients (61.1%) with malignant breast lesions were of 36-49g years old. On the other hand, 30.6%g of patients of over 50 years old were found to have malignant breast lesions. However, 8.3%g of malignant breast lesions were observed in women of less than 35g years old and also agree with study (Hatim *et al*,2017) show

**Table(4-2) bacterial diagnosis test for bacteria associated with breast tumor tissue**

| Test | *S. warneri* | *S. aureus* | *K. pneumoniae* | *S. marcescns* | *E. coli* | *P. mirabilis* | *p. auroginosa* |
|---|---|---|---|---|---|---|---|
| Gram stain | + | + | - | - | - | - | - |
| Spore forming | - | - | - | - | - | - | - |
| Catalase | + | + | + | + | + | + | + |
| Oxidase | - | - | - | - | - | - | + |
| Coagulase | - | + | - | - | - | - | - |
| Motility | - | - | - | + | + | + | + |
| Urease | - | + | + | + | - | + | - |
| Indole | - | - | - | - | + | - | - |
| Methyle red | - | + | - | - | + | + | - |
| Vogas proscure | - | + | + | + | - | - | - |
| Cimon citrate | - | + | + | + | - | + | + |

**Table (4-3) bacterial in types of breast tumors**

| Types of tumors | Types of bacteria with percent % | | | | | | |
|---|---|---|---|---|---|---|---|
| | ***S. Aureus*** | ***K. pneumoniae*** | ***S. warneri*** | ***S. marcescns*** | ***E. coli*** | ***P. mirabilis*** | *p. auroginosa* |
| **Malignant breast tumor** | 68% | 29% | 33% | 57% | 46% | 67% | 58% |
| **benign breast tumor** | 32% | 71% | 67% | 43% | 54% | 33% | 42% |

**Table(4-4)Comparison in CA15-3 concentration between malignant, benign breast tumors and control**

| Before chemotherapy | | | | |
|---|---|---|---|---|
| **Parameter** | **M±SD concentration pg/ml** | | | **P value** |
| | **Malignant** | **Benign** | **Control** | |
| **CA15-3** | 109.349±35.504 | 104.896±25.623 | 62.802±17.598 | **0.000*** ** |

*(p≤0.05) is considered significant

In table (4-4)g the mean of malignant breast tumors was 109.349g ,the mean of Benign breast tumors was 104.896 compared with control the mean was 62.802 the result showed that concentration of cancer antigen CA 15-3 increase significant malignant breast tumors and Benign breast tumors than control these study was agree with study in Baghdad (Hashim,2014

And this study was agree with study (Eskelinen *et al*.,1988 ) who showed that

The result of study concentration of CA 15-3g in benign breast tumors was significant increased compare with control this study was dis agree with study in Baghdad (Hashim ,2014 )g show that normal CA 15-g 3

**Table (4-5) Comparison between patients and control with breast tumors before chemotherapy**

| Before chemotherapy | | | |
|---|---|---|---|
| **Parameter** | **M±SD concentration pg/ml** | | **P value** |
| | **Patients** | **Control** | |
| **CA15-3** | 106.307±28.881 | 62.802±17.629 | **0.000*** ** |

*(p≤0.05) is considered significant

**Table (4-6) Effect of age group on concentration of CA15-3 in patients with breast tumors**

| **Age groups Year** | **Concentration pg/ml M±SD** | **P value** |
|---|---|---|
| | | |
| (14-34) | 113.992±47.201 a | **0.9** |
| (35-55) | 113.994±55.440 a | |
| (56-65) | 128.847±36.452 a | |

*(p≤0.05) is considered significant

* **Duncan test**

In table (4-6) showed that the effect of age on the concentration of CA15-3 , the mean of first age group was 113.992, in second age group was 113.994 and the mean of third age group was 128.847 the result was no significantly affect the age group on the concentration of CA15-3 this

study was agree with study (Khadhum *et al*.,2022)g in showed that that there is no significant difference (P >g 0.05)

The present study was disagree with (Othman *et al*.,2018 ) who showed

**Table (4-7) Comparison between patients and control on CA15-3 with breast tumors after chemotherapy**

| After chemotherapy | | | |
|---|---|---|---|
| **Parameter** | **M±SD concentration pg/ml** | | **P value** |
| | **Patients** | **Control** | |
| **CA 15-3** | 101.107±28.881 | 62.802±28.881 | **0.05*** |

*(p≤ 0.05) is considered significant

In table (4-7) the mean of CA 15-3 in patients with breast cancer was 101.107 increase significantly compare with mean of control group was 62.802thisg study was agree with study (Hasan,2022g ) in who showed

**Table (4-8 ) Comparison in concentration of CA-15-3 between patients before and after chemotherapy**

| **Parameter** | **M±SD concentration pg/ml** | | **P Value** |
|---|---|---|---|
| | **Before** | **After** | |
| **CA15-3** | 106.307±28.881 | 101.107±93.288 | **0.7** |

*(p≤ 0.05) is considered significant

In table (4-8)g show that concentration of CA 15-3g before and after was no significant difference these study was disagree with study (Gupta *et al*.,2018)

**Table (4-9 )Comparison in CA15-3 concentration between malignant and benign breast tumors**

| Parameter | Concentration of CA15-3 | | P Value |
|---|---|---|---|
| | M±SD pg/ml | | |
| | benign breast tumors | Malignant breast tumors | |
| **CA15-3** | 101.765±28.752 | 118.799±26.153 | **0.7** |

*(p≤ 0.05) is considered significant

**4-4 Immunological study**

**4-4-1 Interleukins 1 alphas , Interleukind 1 betas and TLR2 cytokines detection**

**Table (4-10) Concentration of systemic TLR2 , IL1 alpha and IL1 beta between patients and control in blood**

| Parameters | M±SD concentration pg/ml | | P- value |
|---|---|---|---|
| | Patients | Control | |
| **TLR2 systemic** | 9.953±4.606 | 6.774±3.855 | 0.04* |
| **IL1 beta systemic** | 5.599±3.550 | 3.640±1.996 | 0.04* |
| **IL1 alpha systemic** | 1.302±0.912 | 0.617±0.240 | 0.003** |

*(p≤ 0.05) is considered significant

In table (4-10) show that concentration of IL1 α in serum patients with mean was 9.953 increase significantly compare with control with mean 6.774g this study was agree with study (Al-Hassan *et al*.,2012g )g

In table (4-10)g showed that the concentration of IL1β in serum patient with mean 5.599 was increase significantly compare with control with mean 3.640g this study was agree with study in Erbil (Mohammed, and

Qadir , 2023) also this study was agree with study in china (Wang *et al*.,2019) who showed that

This study also agree with study in Kirkuk (Sulaiman *et al.*,2019) city who show that

In table ( 4-10) showed that concentration of TLR2 in patients with breast tumors with mean 9.953g was increase significantly compare with control group with mean 6.774, this study was agree with study (EL-kharashy *et al*.,2021) who showed that Ag This study was agree with study (Abdulabbas and Shani,2022) in Basra who showed that the serum levels

This study was disagree with study (Al-Ammiri and, Al-Derzi ,2013) who showed that

**Table (4-11) concentration of IL1α, Ilβ and TLR2 in patients group and healthy**

| Parameters | Concentration pg/ml | | | P value |
|---|---|---|---|---|
| | Mg ±SD | | | |
| | Breast cancer | Benign tumor | Healthy | |
| **IL1 α** | 0.891 ± .288 ab | 1.208 ±0.589 b | 0.616 ± 0.239 a | 0.001** |
| **IL1β** | 4.630 ± 2.434 a | 4.270 ± 2.204 a | 3.640 ± 1.996 a | 0.5 |
| **TLR2** | 11.826 ± 4.305 b | 8.967 ± 4.556 ab | 6.773 ± 3.855 a | 0.03* |

In table (4-11) show that concentration of IL1α in malignant breast with mean was 0.891 , in benign breast tumors the mean was 1.208g but in healthy with mean 0.616 ,

This study show that the mean of TLR2 was increase significantly in malignant breast tumors this study was agree with study ( Al- ammiri and Al-Derzi ,2013 ) who show that :

In this study the mean of TLR2g in benign breast tumors was 8.967 no significant increase these study was disagree with study (Al-g ammiri and Al-Derzig ,2013)

| Parameters / Age/years | concentration pg/ml ( M±SD) | | | P-g value |
|---|---|---|---|---|
| | 14-29 | 30-45 | 46-66 | |
| **TLR2 systemic** | 9.632±4.472 | 11.9131±3.040 | 7.710±5.869 | 0.1 |
| **TLR2tissue** | 0.184±0.084 | 0.251±0.175 | 0.160±0.114 | 0.3 |
| **IL1 beta systemic** | 4.895±2.710 | 6.254±4.010 | 6.433±4.960 | 0.6 |
| **IL1 beta tissue** | 20.833±12.215 | 20.333±17.643 | 22.619±9.567 | 0.9 |

**(4-12) Systemic and Local of TLR2 , IL1 beta and IL1 alpha in Patients with Breast Tumors According to Age Groups**

| | | | | |
|---|---|---|---|---|
| **IL1 alpha systemic** | 1.268±0.756 | 1.064±0.485 | 1.171±0.463 | 0.7 |
| **IL1 alpha tissue** | 1.679±0.780 | 0.626±0.338 | 1.444±0.825 | 0.002** |

In table (4-12)g the result of this study 0.626g but in third age group with mean 1.444 the result shows significant different in the concentration of IL1g α between age group, but in serum the mean of IL α in first age group was 1.268 , in second group the mean was 1.064 and in third age group was1.171 the result no significant different between age groups, IL1 β in patients serum in first age group was 4.895 , in second group the mean was 6.254andg in third age group was 6.433 the result no significant different between age groups , IL1 β in patients tissue in first age group was 20.833, in second group the mean was 20.333 and in third age group was 22.619 the result no significant different between age groups ,

TLR2g in patients serum in first age group was 9.632 , in second group the mean was 11.9131andg in third age group was 7.710the result no significant different between age groups , TLR2 in patients tissue in first age group was 0.184, in second group the mean was 0.251andg in third age group was 0.160 the result no significant different between age groups this agree with study (Abdulabbas and Shani,2022g ) who showed that

The present study showed that no significant different in concentration of IL1 beta in patient serum between age group this study was agree with study (Lafrenie *et al*.,2023) who showed that no significant between age group and IL1 beta concentration

**Table (4-13) concentration of IL1α ,g IL1 β and TLR2 between patients before and after chemotherapy**

| Parameters | Concentration pg/ml M±SD | | P_ value |
|---|---|---|---|
| | Before chemotherapy | After chemotherapy | |
| **IL1α** | 1.301±0.913 | 0.619±0.323 | 0.03* |
| **IL1β** | 5.598±3.550 | 17.593±16.354 | 0.001* |
| **TLR2** | 9.953±4.606 | 7.774±2.674 | 0.2 |

*(p≤0.05) is considered significant

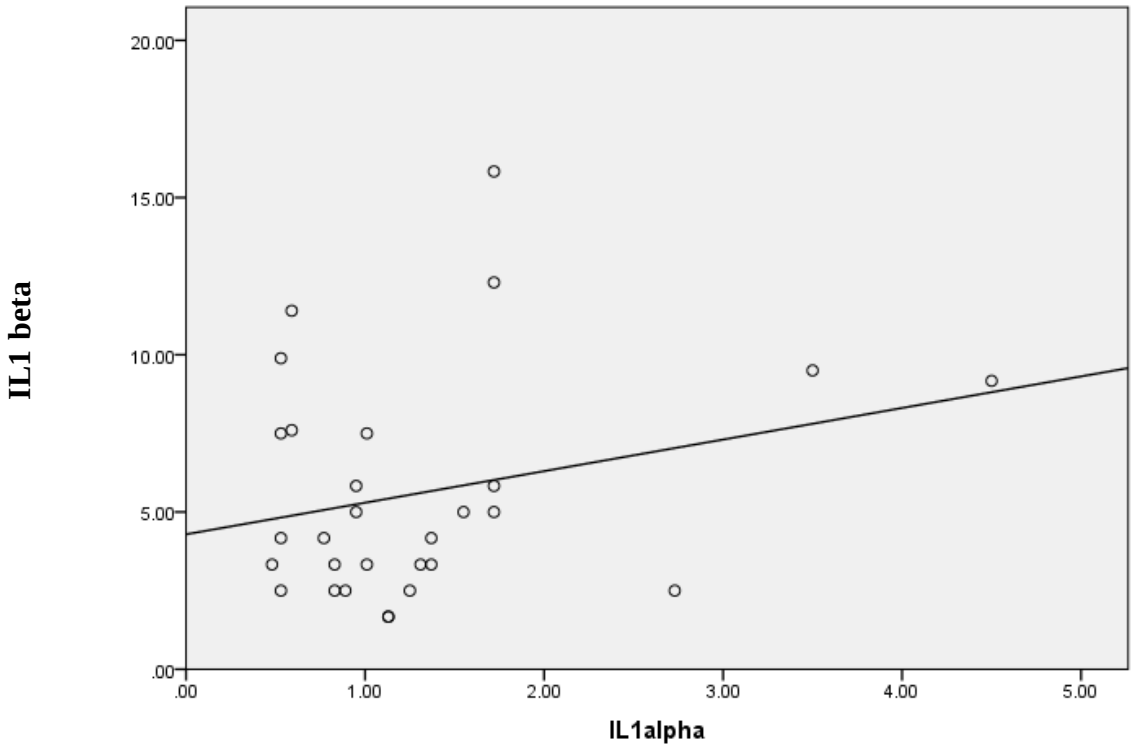
In table (4-13) showed that the of IL1 α in patients without chemotherapy was 1.301 but mean of IL1 α with chemotherapy was 0.619 were significant different between , the mean IL1 β patients without chemotherapy was 5.598g but mean of IL β with chemotherapy was 17.593, but the mean of TLR2 without chemotherapy was 9.953 but mean of TLR2 after chemotherapy was 7.774 .this study showed that increase significantly of IL1α and IL1β without and with chemotherapy this study was agree with study (Tsavaris *et al*.,2022) who showed that

Also disagree with study (Felix *et al*.,2018 )

**Table (4-14)Comparison between concentration of TLR2 , IL1 alpha and IL1 beta between patients in blood and tissue patients**

| Parameters | M±SD | | P- value |
|---|---|---|---|
| | Blood | Tissue | |
| **TLR2** | 9.953±4.606 | 0.209±0.143 | 0.000*** |
| **IL1 beta** | 5.599±3.550 | 21.000±14.356 | 0.001** |
| **IL1 alpha** | 1.304±0.913 | 1.086±0.818 | o.4 |

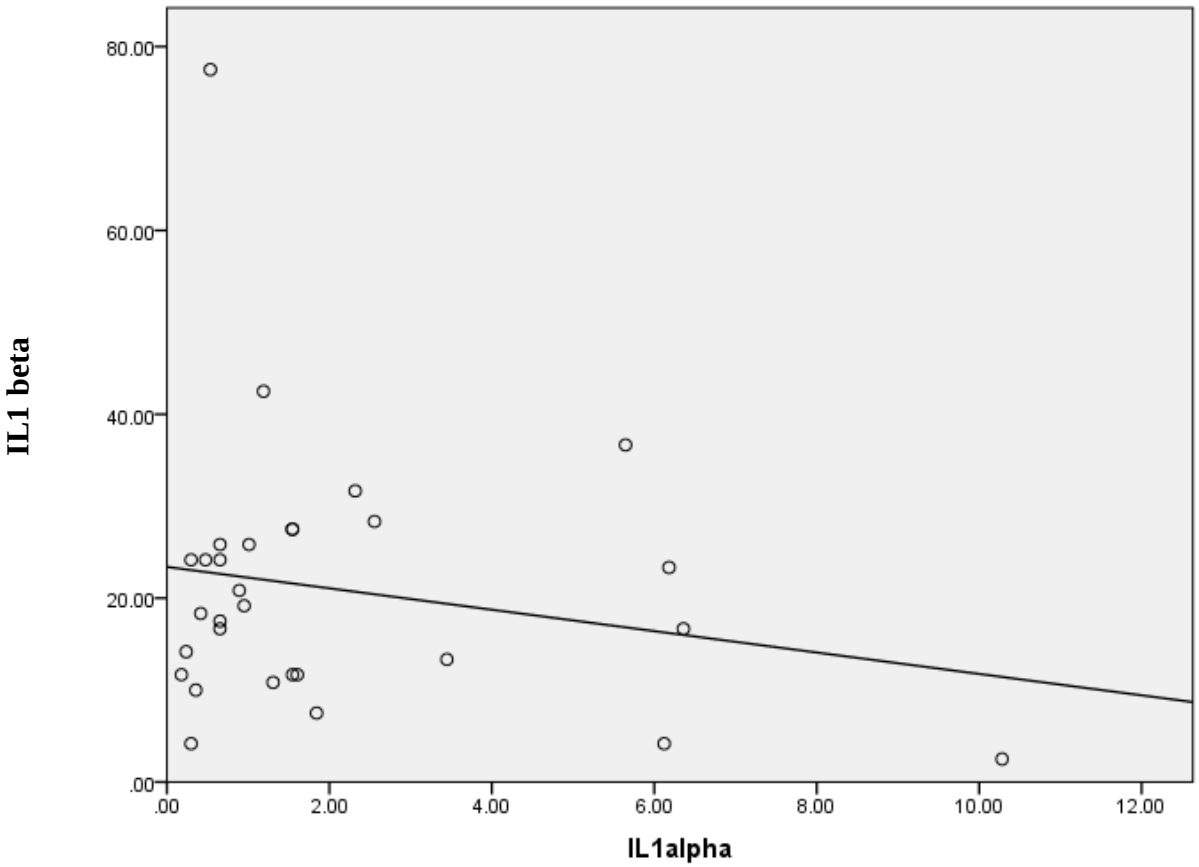
*(p≤ 0.05) is considered significant




P=0.1

R=0.3

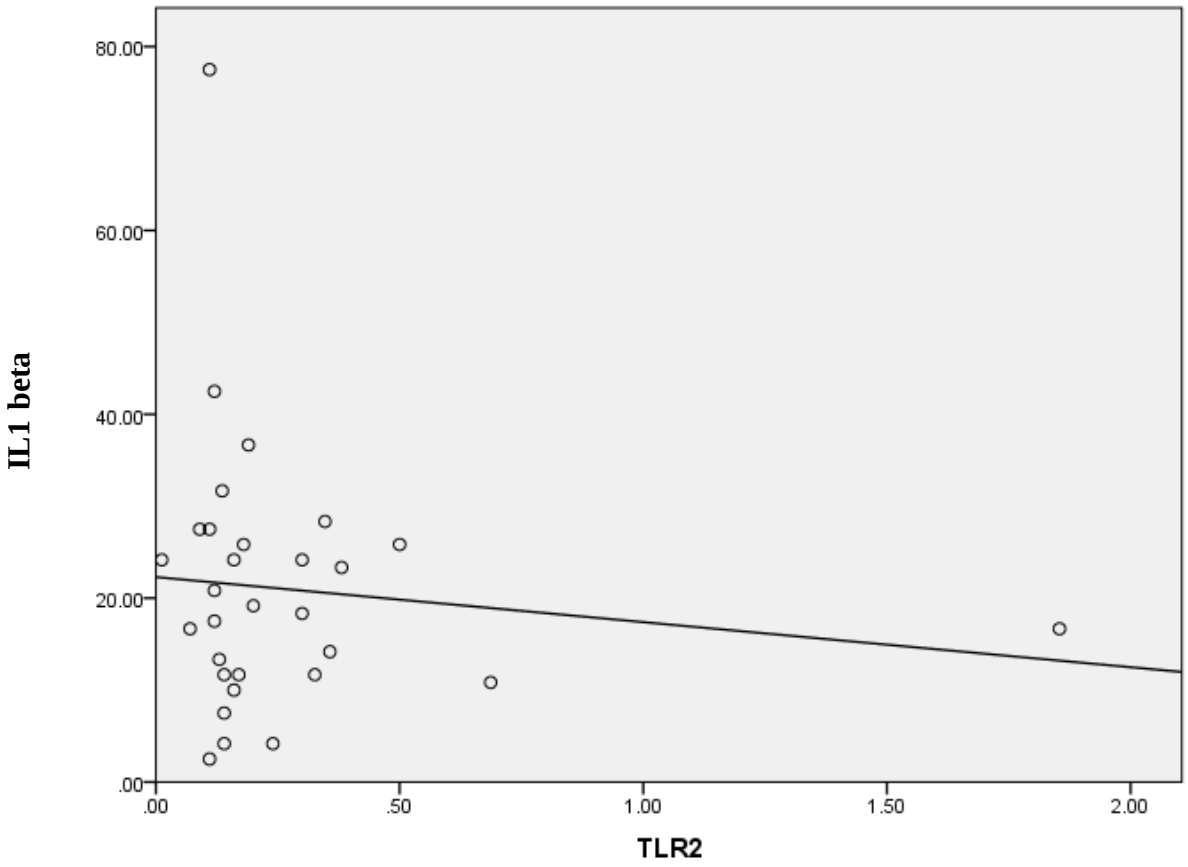
**Figure (4-3)Correlation between IL1 BETA and IL1 alpha blood**




P= 0.3

R=-0.2

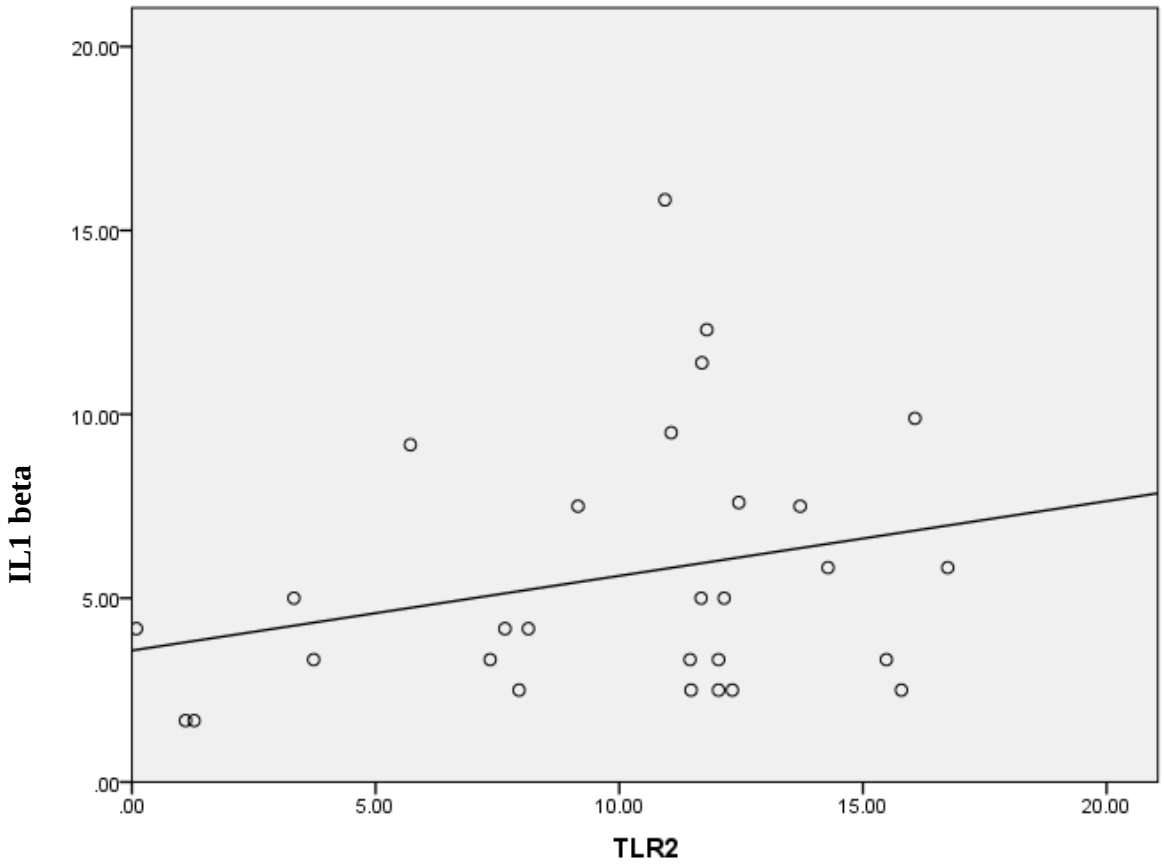
**Figure (4-4) Correlation between IL1 beta and IL1 alpha mucosal**




P= 0.5

R=-0.1

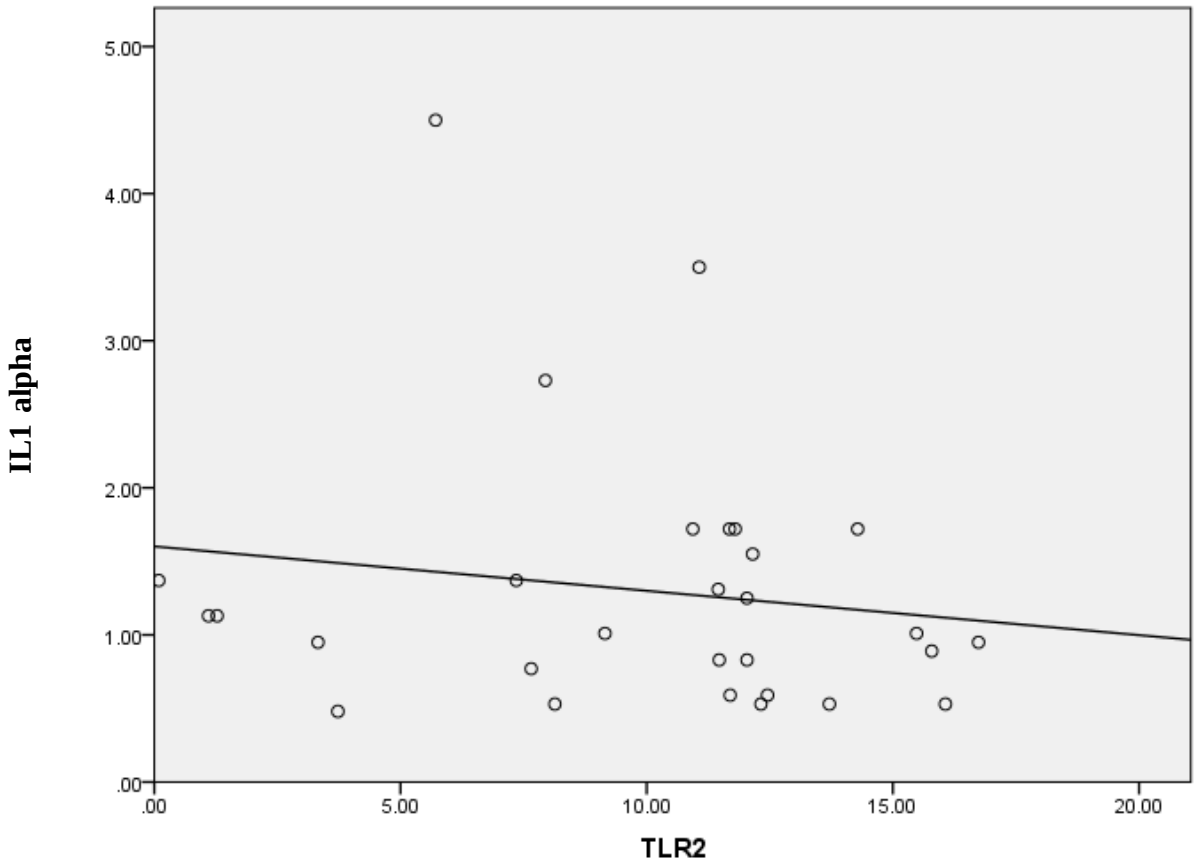
**Figure (4-5) Correlation between TLR2 and IL1 BETA in tissue**




P= 0.1

R=0.3

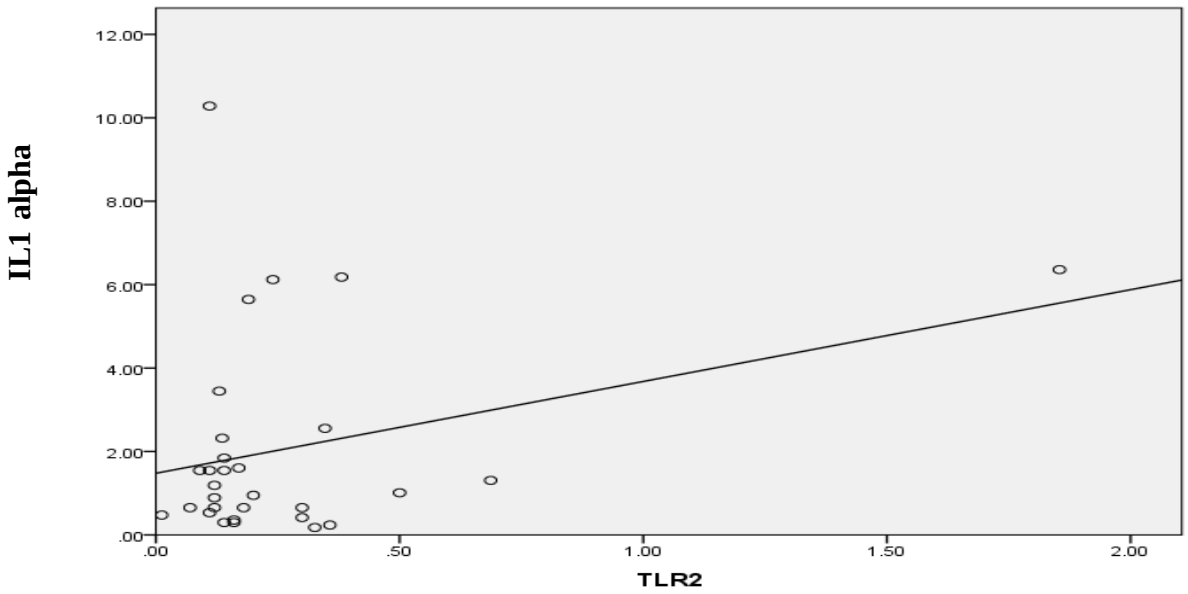
**Figure (4-6) Correlation between TLR2 and IL1 beta in blood**




P=0.4

R=-0.2

**Figure (4-7) Correlation between TLR2 and IL1 alpha in blood**




P= 0.1

R=0.3

**Figure (4-8) Correlation between TLR2 and IL1 alpha in tissueg**

## 4-5 Molecular study of breast tumors

### 4-5-1 IL-1 alpha -889 C>T promoter primer

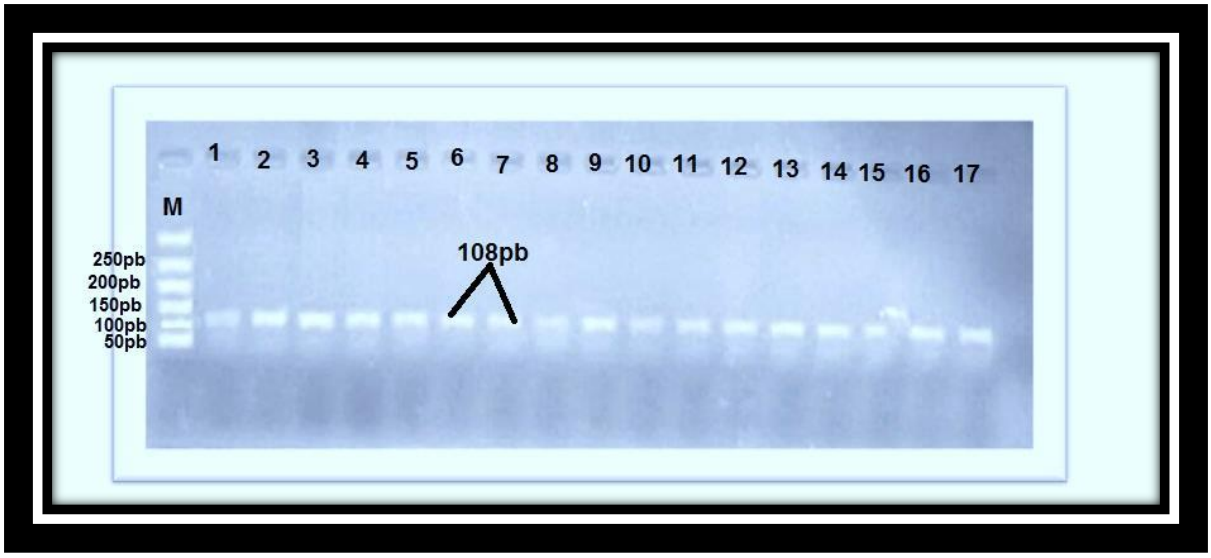



**Figure (4-9) Electrophoreses pattern of PCR product of IL-1 alpha -889 C>T in blood, M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 53.9**

PCR product of IL-1 alpha -889 C>T gene was amplified by using specific primer . the PCR product (band ) of IL-1 alpha -889gC>T gene was 108 –bp in tissue patients figure (4-10) .

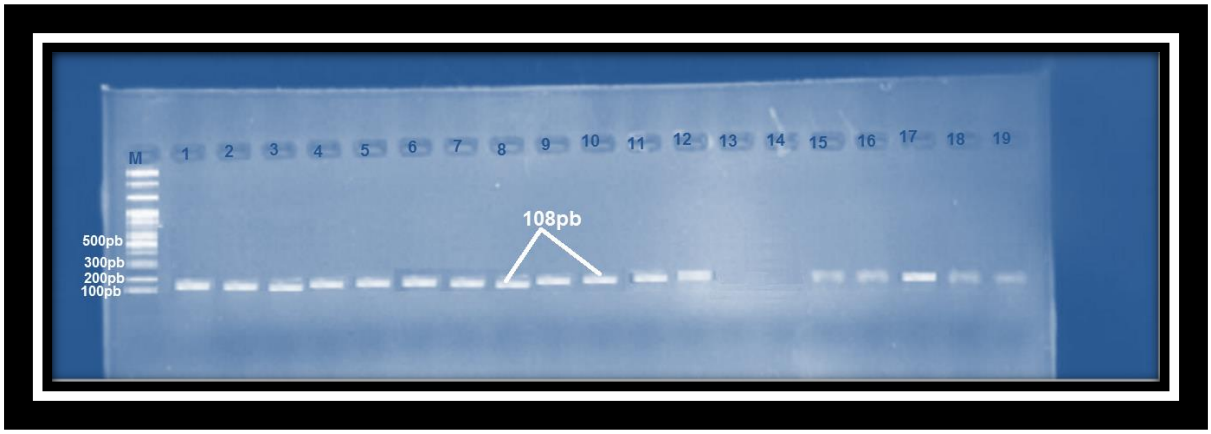



**Figure (4-10) Electrophoreses pattern of PCR product of IL-1 alpha -889 C>T in tissue , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 53.9**

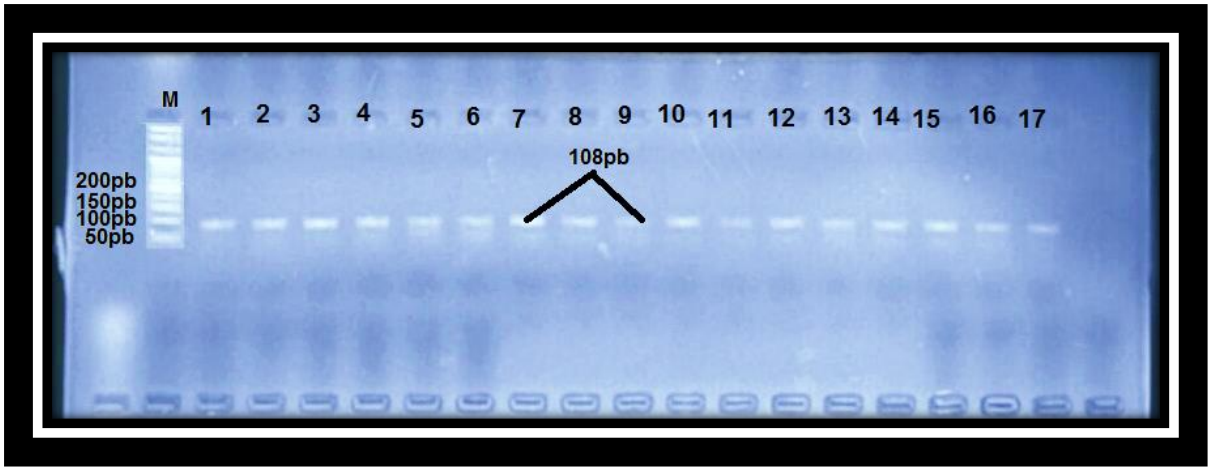



**Figure (4-11) Electrophoreses pattern of PCR product of IL-1 alpha -889 C>T in blood of chemotherapy , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 53.9**

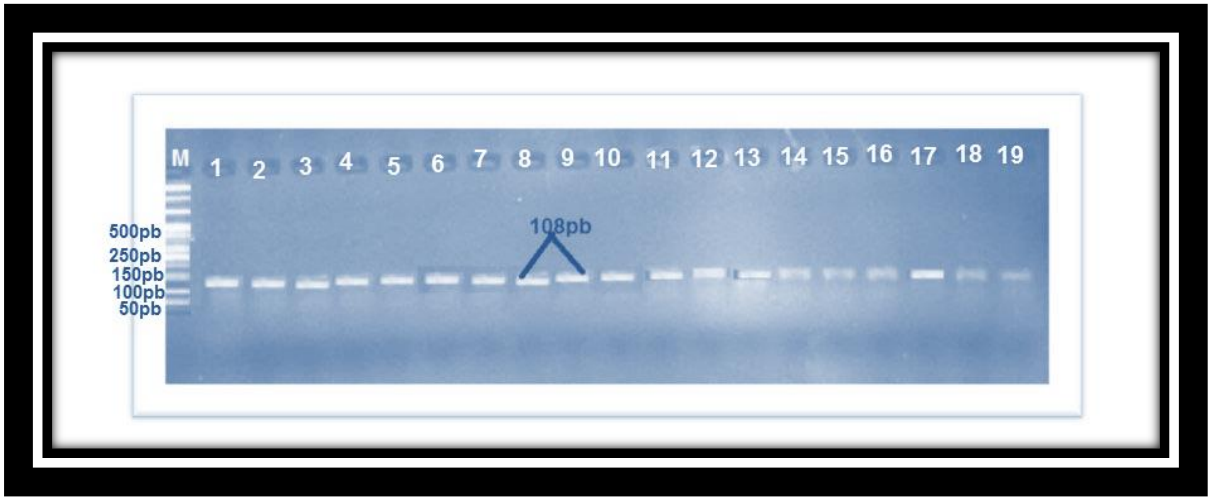


**Figure (4-12) Electrophoreses pattern of PCR product of IL-1 alpha -889 C>T blood control , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 53.9**

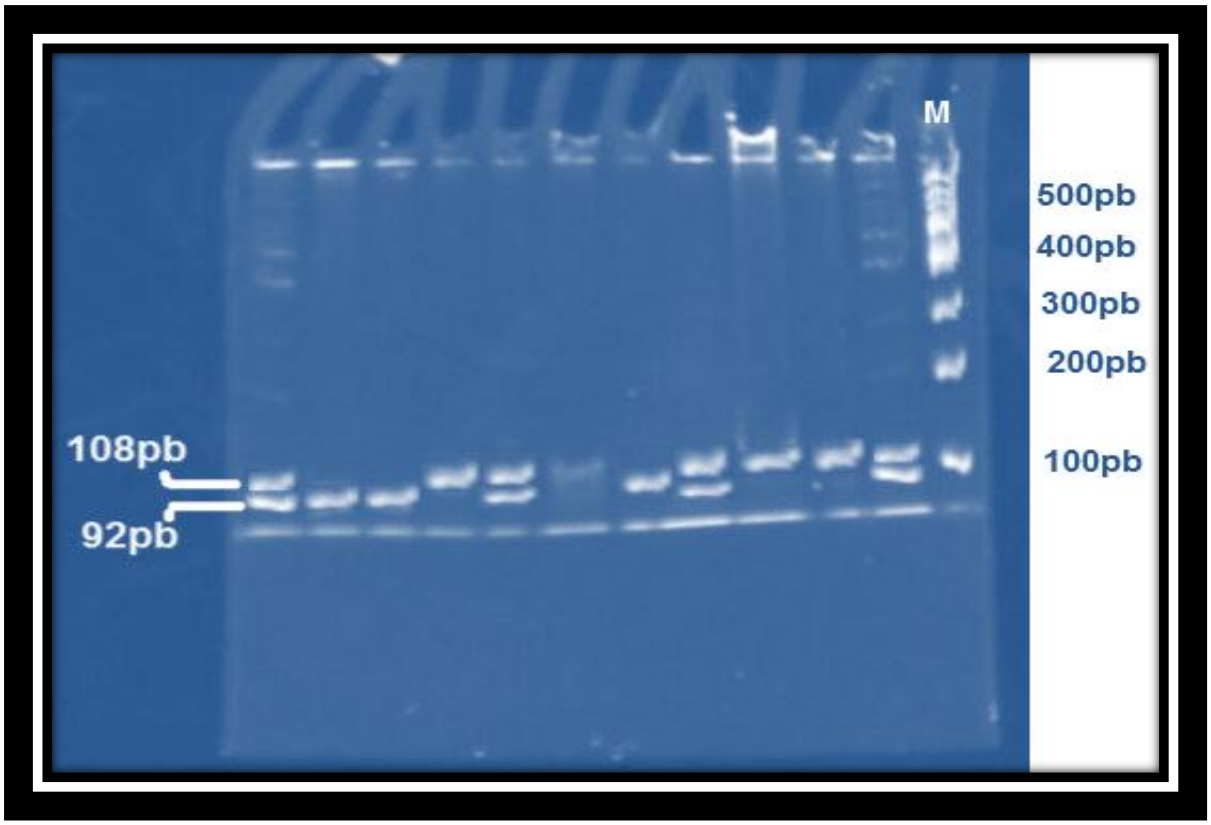



**figure ( 4-13 ) electrophorases patteren of IL-1 alpha -889 C>T gene PCR-RFLP by PAGE gel for PCR product (108pb) with restriction enzyme *NocI* . M : DNA ladder . lane (5,9,10) homozygote TT genotype 92pb , lane (2,3,8) homozygote (CC) genotype (108pb) lane (1,4,6,7,11) heterozygote (CT) genotype (108pb and 92 pb)**

%)and 25(25%) in blood patients ,where it were 17(34%), 20(40%) and 13(26%) in healthy groups table (4-15) .the P-value of the each genotypes frequencies of IL-1 alpha -889 C>T gene were nosignificant 0.57 and 0.47 for CT and TT respectively , patients with genotype CT were

affected by breast tumors approximately one time comparsion with patients having genotype TT (odd ratio = 1.28 and 0.72 .

**Table (4-15)genotype frequency of IL-1 alphas -889 C>T gene polymorphism with allele frequency in healthy control and blood breast tumors patients**

| **Genotype IL-1g alpha -889 C>T** | **Blood patients** | **Healthy (control )** | **P- value** | **Odd ratio** |
|---|---|---|---|---|
| **CC** | 25(25%) | 13(26%) | | |
| **CT** | 30(30% | 20(40% | 0.57 | **1.28(0.53- 3.08)** |
| **TT** | 45(45%) | 17(34%) | 0.47 | **0.72( 0.30-1.73)** |
| **Total number** | 100 | 50 | | |
| **Allele frequency** | | | | |
| **C** | 80(0.4) | 46(0.54) | | |
| **T** | 120(0.6) | 54(0.46) | 0.32 | **0.78( 0.48-1.27)** |

In tissue patient C>T gene polymorphism where it were 15(21%), 30(30%) and 25(36%) in the breast tumors patients , table (4-16) . CT patients with genotype CT were affected by breast tumors approximately one time comparison with patients having genotype TT (odd ratio = 1.00 and 0.33) .

**Table (4-16)genotype frequency of gene polymorphism with allele frequency in blood patients and tissue breast tumors patients**

| Genotype IL-1 alpha -889 C>T | blood patients | Tissue patients | P- value | Odd ratio |
|---|---|---|---|---|
| **CC** | 25(25%) | 25(36%) | | |
| **CT** | 30(30%) | 30(30%) | 1.00 | 1.00(047-2.11) |
| **TT** | 45(45%) | 15(21%) | 0.007* | 0.33(0.14- 0.74) |
| **Total number** | 100 | 70 | | |
| **Allele frequency** | | | | |
| **C** | 80(0.4) | 80(0.57) | | |
| **T** | 120(0.6) | 60(0.4) | 0.002* | 0.50(0.32- 0.77) |

in chemotherapy patients table (4-17)in the breast tumors patients the P-value of the genotypes frequencies of IL-1 alpha -889 C>T gene were significantly 0.007 for TT ($p<0.05$) and no significant for CC and CT patients with genotype TT were affected by breast tumors approximately one time comparison with patients having genotype CT (odd ratio = 1.50g and 0.91)

**Table (4-17)genotype frequency of gene polymorphism with allele frequency in healthy control and blood breast tumors patients**

| Genotype IL-1 | Chemotherapy | Healthy | P value | OR |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| **alpha -889 C>T** | | | | **CL 95%** |
| **CC** | 10(33%) | 16(32%) | | |
| **CT** | 15(50%) | 22(44%) | 0.86 | 0.91( 0.32-2.56) |
| **TT** | 5(17%) | 12(24%) | 0.54 | 1.50( 0.40-5.55) |
| **Total** | 30 | 50 | | |
| **Alleles frequency** | | | | |
| **C** | 35(0.58) | 54 | | |
| **T** | 25(0.41) | 46 | 0.59 | 1.19( 0.62- 2.27) |

In blood benign patients table (4-18)in the breast tumors patients the P-value of the genotypes frequencies of IL-1 alpha -889 C>T gene were no significantly where patients with TT more affected by breast tumors comparison with patients having genotype CT(Odd ratio 0.90 , 0.70)

| **Genotype**<br>**IL-1 alpha -889 C>T** | **Malignant**<br>**Blood** | **Benign**<br>**Blood** | **P value** | **OR**<br>**CL 95%** |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| CC | 12(29%) | 20(34%) | | |
| CT | 22(52%) | 26 (45%) | 0.46 | 0.70(0.28-1.76) |
| TT | 8 (19%) | 12 (21%) | 0.85 | 0.90( 0.28-2.83) |
| Total | 42 | 58 | | |
| Alleles | | | | |
| C | 46(0.55) | 66(0.55) | | |
| T | 38(0.45) | 50(0.45) | 0.76 | 0.91( 0.52-1.61) |

**Table (4-18)genotype frequency of gene polymorphism with allele frequency in Malignant and Benign blood breast tumors patients**

In tissue benign patients table (4-19)in the breast tumors patients the P-value of the genotypes frequencies of IL-1 alpha -889 C>T gene were no significantly ,whereas patients with CT more affected by breast tumors comparison with patients having genotype TT(Odd ratio 1.16, 0.98).

**Table (4-19)genotype frequency of gene polymorphism with allele frequency in Malignant and Benign blood breast tumors patients**

| **Genotype IL-1 alpha -889 C>T** | **Malignant Tisuue** | **Benign tissue** | **P value** | **OR CL 95%** |
|---|---|---|---|---|
| CC | 12(50%) | 22(48%) | | |
| CT | 7(29%) | 15(19%) | 0.78 | **1.16( 0.73-3.65)** |

| | | | | |
|---|---|---|---|---|
| TT | 5(21%) | 9(33%) | 0.97 | **0.98( 0.26-3.60)** |
| Total | 24 | 46 | | |
| **Alleles frequency** | | | | |
| C | 31(0.66) | 59(0.64) | | |
| T | 17(0.35) | 33(0.36) | 0.95 | **1.02( 0.49-2.11)** |

### 4-5-2 IL-1 beta C31 T

#### 4-5-2-1 IL-1 beta C31 T genotyping PCR

PCR product of IL-1 beta C31 T gene was amplified by using specific primer . the PCR product (band )


**Figure (4-14) Electrophoreses pattern of PCR product of IL-1 beta C31 T blood patients and control, M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 54.8**

PCR product of IL-1 beta C31 T gene was amplified by using specific primer . the PCR product (band ) of IL-1 beta C31 T gene was 240 –bp in tissue patients figure (4-15) .


**Figure (4-15) Electrophoreses pattern of PCR product of IL-1 beta C31 T tissue patients , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 54.8**

PCR product of IL-1 beta C31 T gene was amplified by using specific primer . the PCR product (band ) IL-1 beta C31 T gene was 240 –bp in chemotherapy patients figure (4-16) .

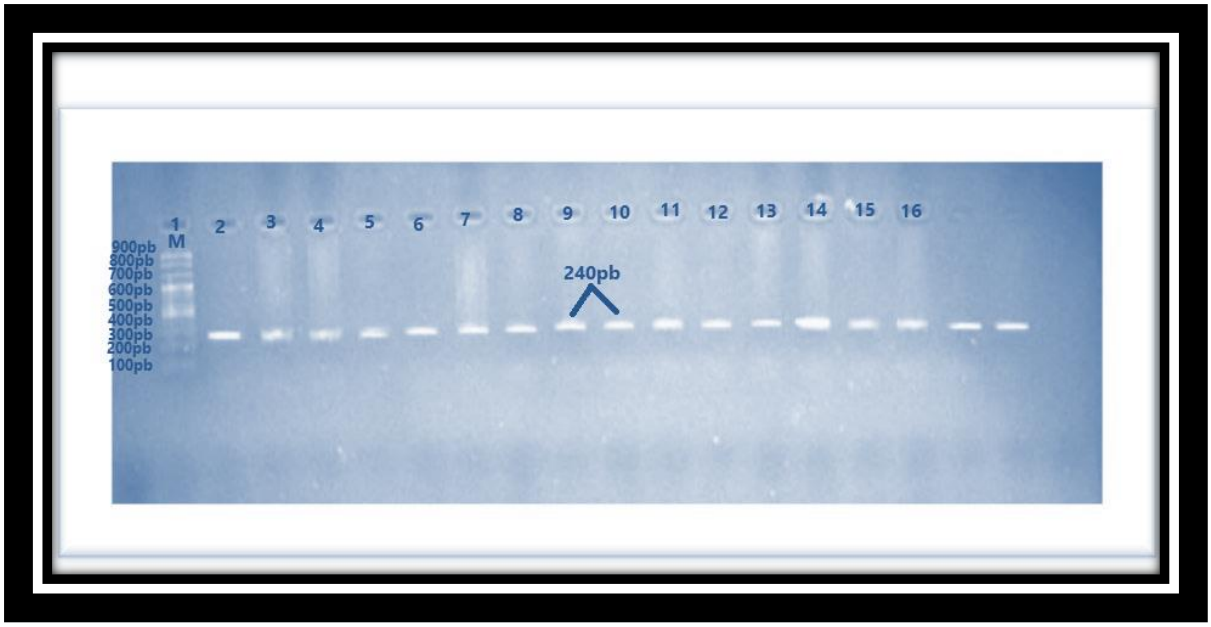
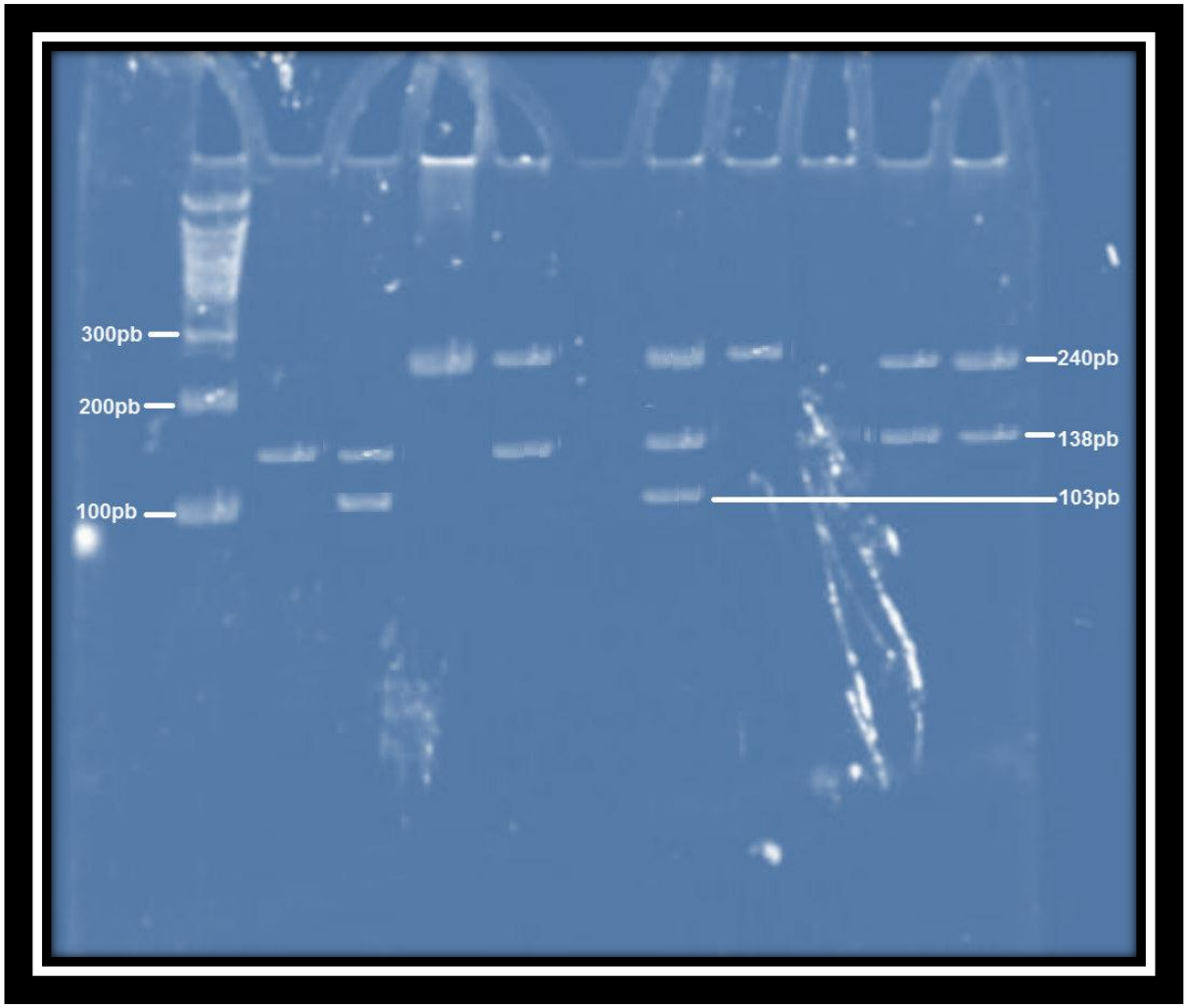



**Figure (4-16) Electrophoreses pattern of PCR product of IL-1 beta C31 T blood chemotherapy , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 54.8**




,where it were 17(34%) , 20(40%)and 13(26%)in healthy groups table (4-20) . by breast tumors approximately comparison with patients having genotype CT (odd ratio = 0.93 and 0.59 ).in present study CC and CT not related with breast cancer this agree with study in turkey ( Eras *et al*.,2019),who showed that CT heterozygote genotype was not related with breast cancer ,

**Tablg (4-20)Genotype of IL-1 beta C31 T gene polymorphism with allele frequency in patients' blood and healthy**

| Genotype IL-1 beta C31 T | Patients (blood) | Healthy | P value | OR<br>CL 95% |
|---|---|---|---|---|
| CC | 20(20%) | 13(26%) | | |

| | | | | |
|---|---|---|---|---|
| **CT** | 52(52%) | 20(40%) | 0.23 | 0.59( 0.24-1.41) |
| **TT** | 28(28%) | 17(34%) | 0.88 | 0.93( 0.37-2.34) |
| **Total** | 100 | 50 | | |
| **Alleles frequency** | | | | |
| **C** | 92(0.46) | 46(0.46) | | |
| **T** | 108(0.54) | 54(0.54) | 1.00 | 1.00( 0.61-1.61) |

In tissue patient table (4-21) .the P-value of the genotypes frequencies of IL-1 beta C31 T gene were no significantly for TT ($p<0.05$) and no significant for CC and CT patients with genotype CT were affected by breast tumors approximately one time comparison with patients having genotype TT (odd ratio = 0.62 and 0.58) .

**Table (4-21)Genotype of IL-1 beta C31 T gene polymorphism with allele frequency in patients' blood and tissue**

| Genotype IL-1 beta C31 T | Patients (Blood ) | Patients (tissue ) | P value | OR CL 95% |
|---|---|---|---|---|
| **CC** | 20(20%) | 7(23%) | | |
| **CT** | 52(52%) | 14(47%) | 0.62 | 0.76( 0.27-2.18) |
| **TT** | 28(28%) | 7(23%) | 0.58 | 0.71( 0.21-2.35) |
| **Total** | 100 | 28 | | |
| **Alleles frequency** | | | | |
| C | 92(0.46) | 28(0.56) | | |
| T | 108(54) | 28(0.44) | 0.77 | 0.85( 0.28- 2.51) |

In chemotherapy beta C31 T gene were no significantly for TT and no significant for CC and CT patients with genotype TT were affected by breast tumors approximately one time comparison with patients having genotype CT (odd ratio = 1.01 and 0.76) .

**Table (4-22)Genotype of IL-1 beta C31 T gene polymorphism with allele frequency in patients with chemotherapy and healthy**

| **Genotype IL-1 beta C31 T** | **(Chemotherapy )** | **Healthy** | **P value** | **OR CL 95%** |
|---|---|---|---|---|
| **CC** | 7(23%) | 13(26%) | | |
| **CT** | 14(47%) | 20(40%) | 0.65 | 0.76( 0.24-2.41) |
| **TT** | 9(30%) | 17(34%) | 0.97 | 1.01( 0.29-3.45) |
| **Total** | | | | |
| **Frequency Alleles** | | | | |
| **C** | 28(0.47) | 46(0.46) | | |
| **T** | 32(0.53) | 54(0.54) | 0.93 | 1.02(0.54-1.95) |

In blood benign patients (32%) , 12(48%) and 5(20%)table (4-23)in the breast tumors patients the P-value of the genotypes frequencies of IL-1 beta C31 T gene were no significantly where's patients with TT , CT

more affected by breast tumors comparison with patients having genotype CT(Odd ratio 0.31 ,g 0.31).

**Table (4-23)genotype frequency of IL-1 beta C31 T gene polymorphism with allele frequency in Malignant and Benign blood breast tumors patients**

| Genotype IL-1 beta C31 T | Malignant Blood | Benign Blood | P value | OR CL 95% |
|---|---|---|---|---|
| **CC** | 5(20%) | 20(45%) | | |
| **CT** | 12(48%) | 15(33%) | 0.06 | 0.31( 0.09-1.07) |
| **TT** | 8(32%) | 10(22%) | 0.08 | 0.31(0.08-1.20) |
| **Total** | 25 | 45 | | |
| **Alleles frequency** | | | | |
| **C** | 22(0.41) | 55(0.54) | | |
| **T** | 28(0.59) | 35(0.38) | 0.05* | 0.50( 0.24-1.008) |

In tissue benign patients for TT genotype ($p<0.05$) but not significant for CT and CC respectively ,whereas patients with CT more affected by breast tumors comparison with patients having genotype TT(Odd ratio 0.38, 0.26).

**Table (4-24)genotype frequency of IL-1 beta C31 T gene polymorphism with allele frequency in Malignant and Benign blood breast tumors patients**

| Genotype IL-1 beta C31 T | Malignant Tissue | Benign Tissue | P value | ORCL 95% |
|---|---|---|---|---|
| **CC** | 8(17%) | 18(37%) | **g** | |
| **CT** | 23(48%) | 20(42%) | 0.06 | **0.38( 0.13-1.07)** |
| **TT** | 17(35%) | 10(21%) | 0.019* | **0.26( 0.08-0.81)** |
| **Alleles frequency** | | | | |
| **C** | 29(0.44) | 56(0.61) | | |
| **T** | 57(0.56) | 40(0.38) | <0.001* | **0.36(0.19-0.66)** |

,

### 4-5-3 Toll like receptor 2 (TLR2)

### 4-5-3-1TLR2 Asp 299 Gly genotyping PCR

PCR product of TLR2 Asp 299 Gly gene was amplified by using specific primer . the PCR product (band ) of TLR2 Asp 299 Gly gene was 286 – bp in patients and control blood figure (4-18) .

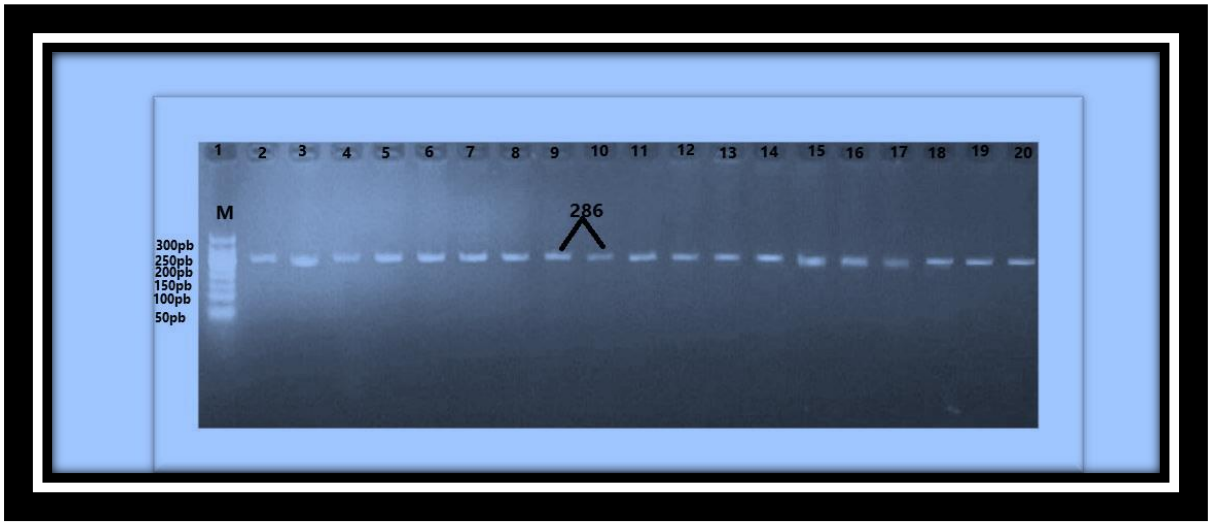


**Figure (4-18) Electrophoreses pattern of PCR product of TLR2 Asp 299 Gly patients and control blood , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 62**

PCR product of TLR2 Asp 299 Gly gene was amplified by using specific primer . the PCR product (band ) TLR2 Asp 299 Gly gene was 286 –bp in chemotherapy patients figure (4-19) .

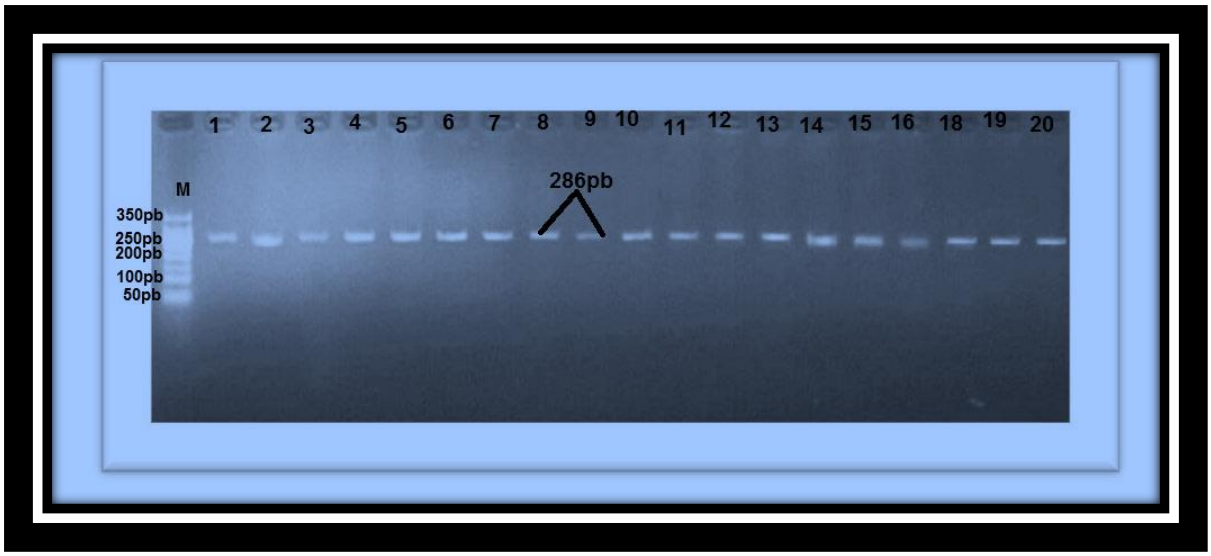



**Figure (4-19) Electrophoreses pattern of PCR product of TLR2 Asp 299 Gly in blood chemotherapy and tissue , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 62**

### 4-5-3-2 DNA sequencing of TLR2 Asp 299 Gly

To prove the result of TLR2 Asp 299 Gly, sequencing were ,the result detection , insertion occur in Guanine , cytosine , thymine and adenine figure (4-20 ).

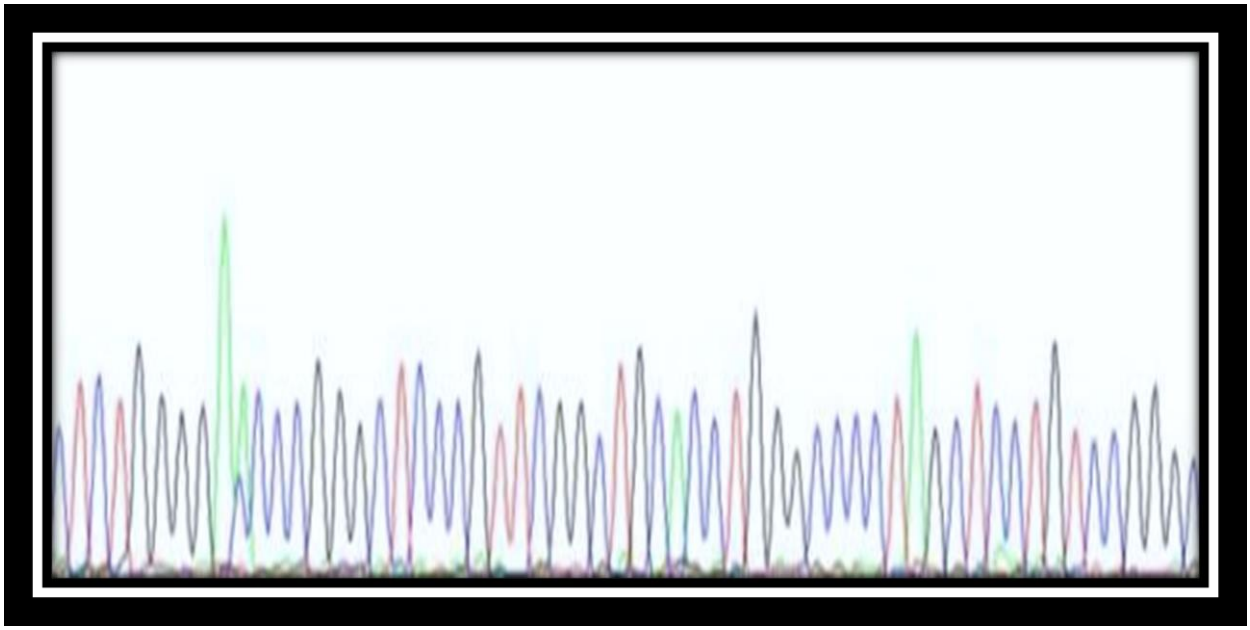


**Figure (4-20) DNA sequencing of TLR2 Asp 299 Gly**

**Table (4-25) number and percentage for nucleotide deletion between patients and control**

| Nucleotide | Deletion | | | |
|---|---|---|---|---|
| | **Number / percentage** | | | |
| | **Patients' blood** | **Healthy (control)** | | |
| **G** | **42(49%)** | **20(43%)** | | |
| **C** | **33(39%)** | **19(41%)** | | |
| **A** | **6(7%)** | **4(9%)** | | |
| **T** | **4(5%)** | **3(7%)** | | |

**Table (4-26) number and percentage for nucleotide deletion between patients' blood and tissue patients**

| Nucleotide | Deletion | | | |
|---|---|---|---|---|
| | **Number / percentage** | | | |
| | **Patients' blood** | **Patients tissue** | | |
| **G** | **42(49%)** | **36(45%)** | | |
| **C** | **33(39%)** | **34(42%)** | | |
| **A** | **6(7%)** | **8(10%)** | | |
| **T** | **4(5%)** | **2(3%)** | | |

**Tableg (4-27)g numberg andg percentageg forg nucleotideg deletiong betweeng patients'g bloodg andg tissueg patients**

| Nucleotide | Deletion | | | |
|---|---|---|---|---|
| | **Numberg /g percentage** | | | |
| | **Chemotherapyg patients** | **Healthyg (control)** | | |
| **G** | **17(55%)** | **20(43%)** | | |
| **C** | **10(32%)** | **19(41%)** | | |
| **A** | **3(10%)** | **4(9%)** | | |
| **T** | **1(3%)** | **3(7%)** | | |

**g**

**Table (4-28) number and percentage for nucleotide deletion between patients and control**

| Nucleotide | Insertion | | | |
|---|---|---|---|---|
| | **Number / percentage** | | | |
| | **Patients' blood** | **Healthy (control)** | | |
| **G** | **12(27%)** | **6(34%)** | | |
| **C** | **11(24%)** | **4(22%)** | | |
| **A** | **6(13%)** | **4(22%)** | | |
| **T** | **16(36%)** | **4(22%)** | | |

**Table (4-29) number and percentage for nucleotide deletion between patients and control**

| Nucleotide | Insertion | | | |
|---|---|---|---|---|
| | **Number / percentage** | | | |
| | **Patients' blood** | **Patients tissue** | | |
| **G** | **12(27%)** | **5(50%)** | | |
| **C** | **11(24%)** | **2(20%)** | | |
| **A** | **6(13%)** | **1(10%)** | | |
| **T** | **16(36%)** | **2(20%)** | | |

**Table (4-30) number and percentage for nucleotide deletion between patients and control**

| Nucleotide | Insertion | | | |
|---|---|---|---|---|
| | **Number / percentage** | | | |
| | **Chemotherapy patients** | **Healthy (control)** | | |
| **G** | **1(8%)** | **6(34%)** | | |
| **C** | **4(31%)** | **4(22%)** | | |
| **A** | **2(15%)** | **4(22%)** | | |
| **T** | **6(46%)** | **4(22%)** | | |

**4**

**4-5-4 -1 TLR4 3725 G / C genotyping PCR**

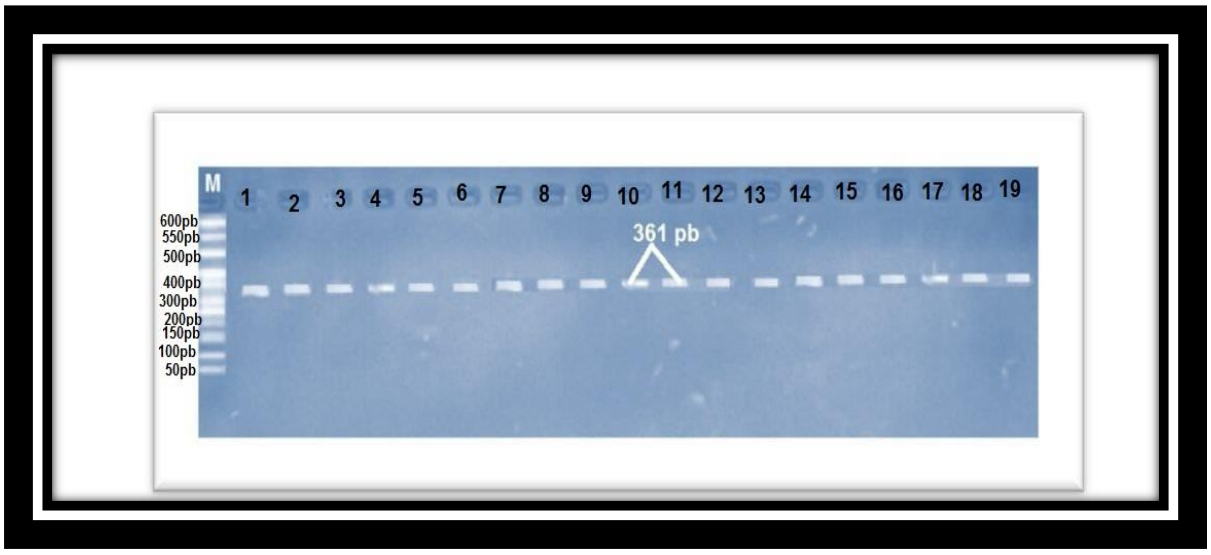


**Figure (4-21) Electrophoreses pattern of PCR product of TLR4 3725 G / C blood patients and control , M : molecular DNA ladder , 1-19 PCR product , the optimum annealing temperature was 57.5**

g PCR product of TLR4 3725 G / C gene was amplified by using specific primer . the PCR product (band ) of TLR4 3725 G /g Cg gene was 361 – bp in tissue patients figure (4-22) .

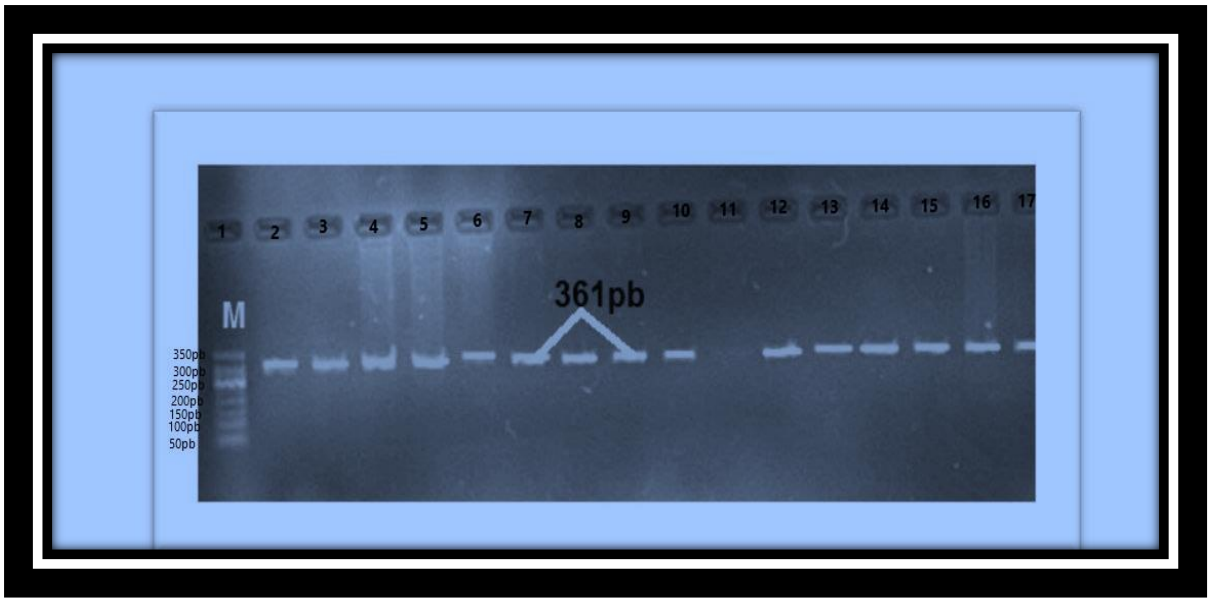



**Figure (4-22) Electrophoreses pattern of PCR product of TLR4 3725 G / C in tissue patients , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 57.5 in tissue patients**

PCR product of TLR4 3725 G / C gene was amplified by using specific primer . the PCR product (band ) TLR4 3725 G / C gene was 361 –bp in chemotherapy patients figure (4-23) .

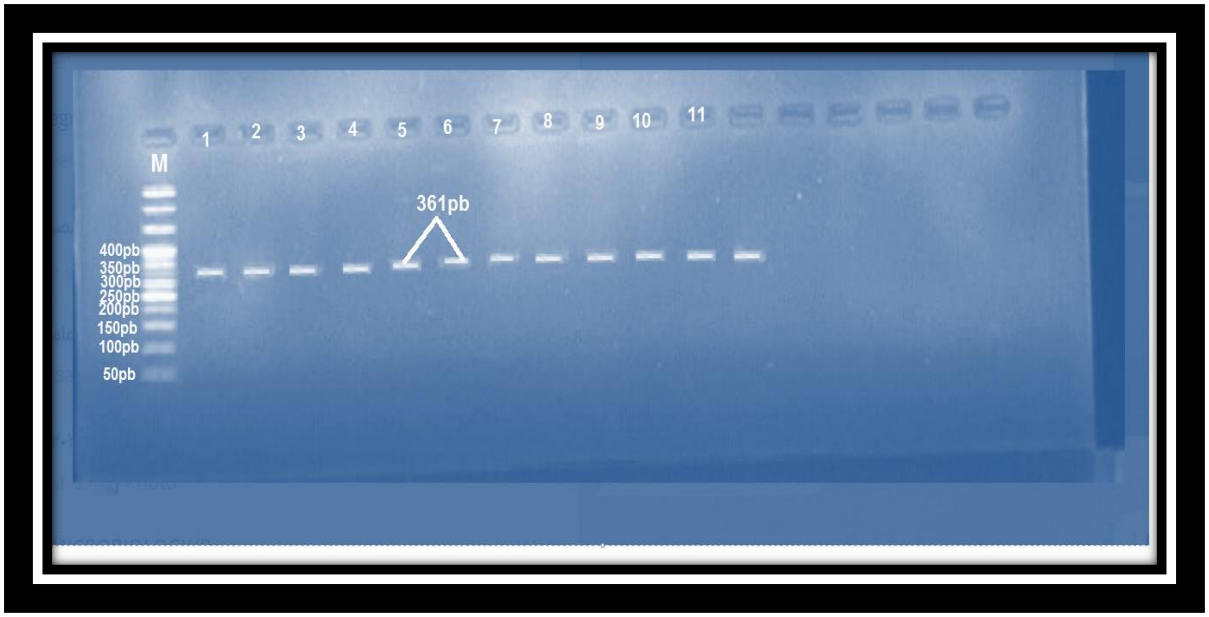



**Figure (4-23)g Electrophoreses pattern of PCR product of TLR4 3725 G / C in blood chemotherapy , M : molecular DNA ladder , 1-17g PCR product , the optimum annealing temperature was 57.5**

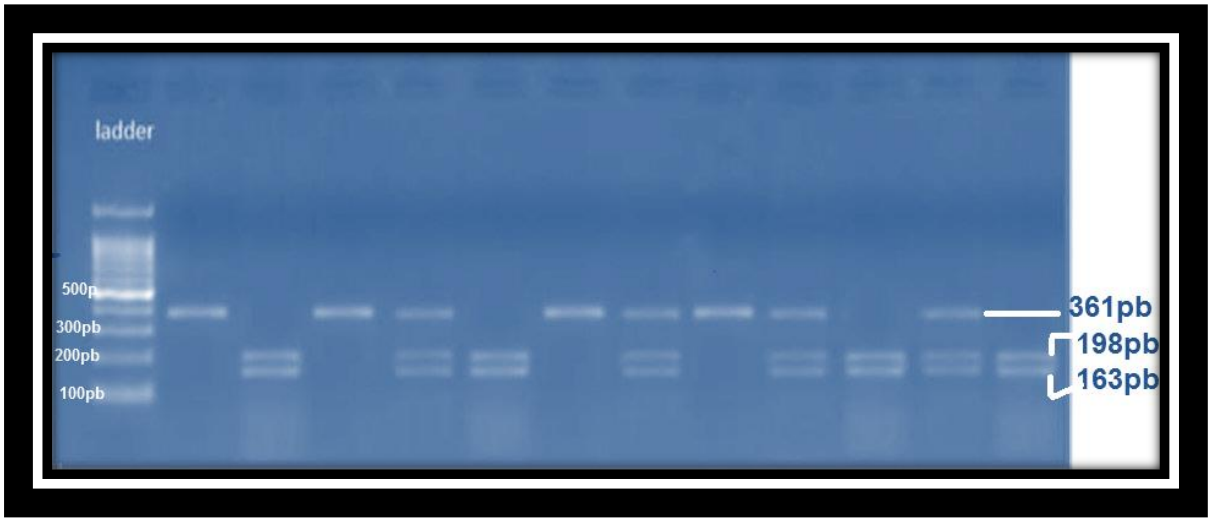



**Figure (g 4-24g )g ) electrophoresis pattern of TLR4 3725 G / C gene**

g

g Genotype, 8(38%) and 1(5%)g in healthy groups table (6) .the P-value of the each genotypes frequencies of TLR4 3725 G / C gene were no significant 0.47 and 0.06 for GC and CC respectively , patients with

genotype GC were affected by breast tumors approximately one time comparison with patients having genotype CC (odd ratio = 1.44 and 0.7) .

**Table (4-31)genotype frequency ofTLR4 3725 G / C gene polymorphism with allele frequency in healthy control and blood breast tumors patients**

| Genotype TLR4 3725 G / C | Blood patients | Healthy | P value | OR CL 95% |
|---|---|---|---|---|
| **GG** | 54(51%) | 12(57%) | | |
| **GC** | 25(24%) | 8(38%) | 0.47 | **1.44(0.52-3.96)** |
| **CC** | 26(25%) | 1(5%) | 0.06 | **0.17( 0.02-1.40)** |
| **Total** | 95 | 21 | | |
| **Alleles frequency** | | | | |
| **G** | 133(0.7) | 32(0.76) | | |
| **C** | 77(0.41) | 10g (0.24) | 0.11 | **0.54( 0.25- 1.15)** |

%) , 15(21%) and 23(33%) in tissue patients ,where it were 54(51%) , 25(24%) and 26(25%)in blood patients groups table (4-32)g .the P-value of the each genotypes frequencies of TLR4g 3725 G / C gene were no significant 0.97 and 0.26 for GC and CC respectively , patients with genotype CC were affected by breast tumors approximately one time comparison with patients having genotype GC (odd ratio = 1.49 and 1.01) .

**Table (4-32)genotype frequency of and tissue breast tumors patients**

| Genotype TLR4 3725 G / C | Blood patients | Tissue patients | P value | OR CL 95% |
|---|---|---|---|---|
| **GG** | 54(51%) | 32(46%) | | |
| **GC** | 25(24%) | 15(21%) | 0.97 | 1.01( 0.45-2.19) |
| **CC** | 26(25%) | 23(33%) | 0.26 | 1.49( 0.73-3.04) |
| **Total** | 95 | 70 | | |
| **Alleles frequency** | | | | |

| | | | | |
|---|---|---|---|---|
| **G** | 133(0.7) | 79(0.56) | | |
| **C** | 77(0.41) | 61(0.44) | 0.19 | 1.33( 0.86-2.06) |

In chemotherapy patients genotype g in healthy groups table (4-33) .the P-value of the each genotypes frequencies of TLR4 3725 G / C gene were no significant 0.49 and 0.20 for GC and CC respectively , patients with genotype GC were affected by breast tumors approximately one time comparison with patients having genotype CC(odd ratio = 0.66 and 0.25) .

**Table (4-33)genotype frequency of TLR4 3725 G / Cg gene polymorphism with allele frequency in healthy control and chemotherapy breast tumors patients**

| **Genotype TLR4 3725 G / C** | **Chemotherapy** | **Healthy** | **P value** | **OR CL 95%** |
|---|---|---|---|---|
| **GG** | 15(50%) | 12(57%) | | |
| **GC** | 10(33%) | 8(38%) | 0.49 | 0.66( 0.21-2.11) |
| **CC** | 5(17%) | 1(5%) | 0.20 | 0.25( 0.02-2.43) |
| **Total** | 25 | 21 | | |
| **Alleles frequency** | | | | |
| **G** | 40(0.8) | 32(0.76) | | |
| **C** | 20(0.4) | 10(0.24) | 0.29 | 0.62( 0.25- 1.52) |

In blood significantly with GC (0.02) , where's patients with GC more affected by breast tumors comparison with patients having genotype CC(Odd ratio 2.77 ,0.55).

**Table (4-34)genotype frequency of**

| Genotype TLR4 3725 G / C | Malignant blood | Benign blood | P value | OR CL 95% |
|---|---|---|---|---|
| **GG** | 20(48%) | 18(34%) | | |
| **GC** | 12(28%) | 30(57%) | 0.02* | 2.77(1.10-6.99) |
| **CC** | 10(24%) | 5(9%) | 0.35 | 0.55( 0.15-1.93) |
| **Total** | 42 | 53 | | |
| **Alleles frequency** | | | | |
| **G** | 52(0.62) | 66(0.62) | | |
| **C** | 32(0.38) | 40(0.38) | 0.96 | 0.85( 0.54-1.77) |

In Tissue benign were 16(67%) , 5(2%) and 3(2%) table (4-35)in the breast tumors patients the P-value of the genotypes frequencies of TLR4 3725 G / C gene were no significantly wherers patients with CC more affected by breast tumors comparison with patients having genotype GC(Odd ratio 3.20 , 2.24).

**Table (4-35) genotype frequency of.**

| Genotype TLR4 3725 G / C | Malignant tissue | Benign tissue | P value | OR CL 95% |
|---|---|---|---|---|
| **GG** | 16(67%) | 20(44%) | | |

| | | | | |
|---|---|---|---|---|
| **GC** | 5(2%) | 14(30%) | g 0.18 | 2.24(0.66-7.54) |
| **CC** | 3(2%) | 12(26%) | g 0.10 | 3.20( 0.76-13-31) |
| **Total** | 24 | 46 | | |
| **Alleles frequency** | | | | |
| **G** | 37(0.77) | 54(0.59) | | |
| **C** | 11(0.23) | 38(0.41) | 0.03* | 2.36( 1.07-5.21) |

## 4-1 Characterization of breast tumors disease

The women with breast tumors divided according to the types of breast tumors age, history of disease , in table ( 4-1) The percentage and number who in age (14-29) years was 48 (28%) , in age (30-45) years was87(51%) and in age (46-66) was 35(21%) this study show the most common infection in woman with breast tumors in age group (30-45)

### Table (4-1) Demographic of subject

| **Patients (170 women)** | | | **Control (50 women)** |
|---|---|---|---|
| Number ( percentage %) | | | |
| Age years | ( 14-29) | 48 (28%) | 18(36%) |
| | (30-45) | 87(51%) | 27(54%) |
| | (46-66) | 35(21%) | 5(10%) |
| Family history | Present | 50(29%) | |
| | Absent | 120(71%) | |
| Status | Married | 104(61%) | |
| | Unmarried | 66(39%) | |
| Types of feeding | Breast feeding | 51(32%) | |
| | Non- breast feeding | 44(27%) | |
| | Mix feeding | 66(41%) | |
| Types of breast tumors | Benign breast tumors | 96(56%) | |
| | Malignant breast tumor | 74 (445%) | |
| Types of Benign breast tumors | Fibrocystic change | 27(28%) | |
| | Fibro adenoma | 49(51%) | |
| | Granulomastitis | 20(21%) | |

This study was disagree with study (Alwan, 2010) also these study was disagree with study (Majid *et al*., 2017)

Age was a risk factor for breast cancer evolution , the may be due to increased chromosomal damages as a result of repeated divided in age increasing, which lead to the accumulation of mutations in the DNA that bring about to cancer development and "the age-related increase in chromosomal harm occurred hurry in women than in men" because the increasing level of aberrations, and rise in the level of X chromosome damage was the main contributor of aging in women (Wojda *et al*., 2006 and Orta and Günebakan, 2012). But this study was agree with study (Uyisenga *et al*.,2020 )

This study was agree with study (Al-Rawi, 2013 ) in Erbil Iraqi showed that.

But this study showed the most common types of breast tumors was benign than malignant this agree with study (Alwan , 2010) in Iraqi who showed that number and percentage of but this study was disagree with study (Al-Rawi, 2013) show that 36 cases of malignant breast lesions fond in studied patients. Most of patients (61.1%) with malignant breast lesions were of 36-49g years old. On the other hand, 30.6%g of patients of over 50 years old were found to have malignant breast lesions. However, 8.3%g of malignant breast lesions were observed in women of less than 35g years old and also agree with study (Hatim *et al*,2017) show

**Table(4-2) bacterial diagnosis test for bacteria associated with breast tumor tissue**

| Test | *S. warneri* | *S. aureus* | *K. pneumoniae* | *S. marcescns* | *E. coli* | *P. mirabilis* | *p. auroginosa* |
|---|---|---|---|---|---|---|---|
| Gram stain | + | + | - | - | - | - | - |
| Spore forming | - | - | - | - | - | - | - |
| Catalase | + | + | + | + | + | + | + |
| Oxidase | - | - | - | - | - | - | + |
| Coagulase | - | + | - | - | - | - | - |
| Motility | - | - | - | + | + | + | + |
| Urease | - | + | + | + | - | + | - |
| Indole | - | - | - | - | + | - | - |
| Methyle red | - | + | - | - | + | + | - |
| Vogas proscure | - | + | + | + | - | - | - |
| Cimon citrate | - | + | + | + | - | + | + |

**Table (4-3) bacterial in types of breast tumors**

| Types of tumors | Types of bacteria with percent % | | | | | | |
|---|---|---|---|---|---|---|---|
| | ***S. Aureus*** | ***K. pneumoniae*** | ***S. warneri*** | ***S. marcescns*** | ***E. coli*** | ***P. mirabilis*** | *p. auroginosa* |
| **Malignant breast tumor** | 68% | 29% | 33% | 57% | 46% | 67% | 58% |
| **benign breast tumor** | 32% | 71% | 67% | 43% | 54% | 33% | 42% |

**Table(4-4)Comparison in CA15-3 concentration between malignant, benign breast tumors and control**

| Before chemotherapy | | | | |
|---|---|---|---|---|
| **Parameter** | **M±SD concentration pg/ml** | | | **P value** |
| | **Malignant** | **Benign** | **Control** | |
| **CA15-3** | 109.349±35.504 | 104.896±25.623 | 62.802±17.598 | **0.000*** ** |

*(p≤0.05) is considered significant

In table (4-4)g the mean of malignant breast tumors was 109.349g ,the mean of Benign breast tumors was 104.896 compared with control the mean was 62.802 the result showed that concentration of cancer antigen CA 15-3 increase significant malignant breast tumors and Benign breast tumors than control these study was agree with study in Baghdad (Hashim,2014

And this study was agree with study (Eskelinen *et al*.,1988 ) who showed that

The result of study concentration of CA 15-3g in benign breast tumors was significant increased compare with control this study was dis agree with study in Baghdad (Hashim ,2014 )g show that normal CA 15-g 3

**Table (4-5) Comparison between patients and control with breast tumors before chemotherapy**

| Before chemotherapy | | | |
|---|---|---|---|
| **Parameter** | **M±SD concentration pg/ml** | | **P value** |
| | **Patients** | **Control** | |
| **CA15-3** | 106.307±28.881 | 62.802±17.629 | **0.000*** ** |

*(p≤0.05) is considered significant

**Table (4-6) Effect of age group on concentration of CA15-3 in patients with breast tumors**

| **Age groups Year** | **Concentration pg/ml M±SD** | **P value** |
|---|---|---|
| | | |
| (14-34) | 113.992±47.201 a | **0.9** |
| (35-55) | 113.994±55.440 a | |
| (56-65) | 128.847±36.452 a | |

*(p≤0.05) is considered significant

* **Duncan test**

In table (4-6) showed that the effect of age on the concentration of CA15-3 , the mean of first age group was 113.992, in second age group was 113.994 and the mean of third age group was 128.847 the result was no significantly affect the age group on the concentration of CA15-3 this

study was agree with study (Khadhum *et al*.,2022)g in showed that that there is no significant difference (P >g 0.05)

The present study was disagree with (Othman *et al*.,2018 ) who showed

**Table (4-7) Comparison between patients and control on CA15-3 with breast tumors after chemotherapy**

| After chemotherapy | | | |
|---|---|---|---|
| **Parameter** | **M±SD concentration pg/ml** | | **P value** |
| | **Patients** | **Control** | |
| **CA 15-3** | 101.107±28.881 | 62.802±28.881 | **0.05*** |

*(p≤ 0.05) is considered significant

In table (4-7) the mean of CA 15-3 in patients with breast cancer was 101.107 increase significantly compare with mean of control group was 62.802thisg study was agree with study (Hasan,2022g ) in who showed

**Table (4-8 ) Comparison in concentration of CA-15-3 between patients before and after chemotherapy**

| **Parameter** | **M±SD concentration pg/ml** | | **P Value** |
|---|---|---|---|
| | **Before** | **After** | |
| **CA15-3** | 106.307±28.881 | 101.107±93.288 | **0.7** |

*(p≤ 0.05) is considered significant

In table (4-8)g show that concentration of CA 15-3g before and after was no significant difference these study was disagree with study (Gupta *et al*.,2018)

**Table (4-9 )Comparison in CA15-3 concentration between malignant and benign breast tumors**

| Parameter | Concentration of CA15-3 | | P Value |
|---|---|---|---|
| | M±SD pg/ml | | |
| | benign breast tumors | Malignant breast tumors | |
| **CA15-3** | 101.765±28.752 | 118.799±26.153 | **0.7** |

*(p≤ 0.05) is considered significant

**4-4 Immunological study**

**4-4-1 Interleukins 1 alphas , Interleukind 1 betas and TLR2 cytokines detection**

**Table (4-10) Concentration of systemic TLR2 , IL1 alpha and IL1 beta between patients and control in blood**

| Parameters | M±SD concentration pg/ml | | P- value |
|---|---|---|---|
| | Patients | Control | |
| **TLR2 systemic** | 9.953±4.606 | 6.774±3.855 | 0.04* |
| **IL1 beta systemic** | 5.599±3.550 | 3.640±1.996 | 0.04* |
| **IL1 alpha systemic** | 1.302±0.912 | 0.617±0.240 | 0.003** |

*(p≤ 0.05) is considered significant

In table (4-10) show that concentration of IL1 α in serum patients with mean was 9.953 increase significantly compare with control with mean 6.774g this study was agree with study (Al-Hassan *et al*.,2012g )g

In table (4-10)g showed that the concentration of IL1β in serum patient with mean 5.599 was increase significantly compare with control with mean 3.640g this study was agree with study in Erbil (Mohammed, and

Qadir , 2023) also this study was agree with study in china (Wang *et al*.,2019) who showed that

This study also agree with study in Kirkuk (Sulaiman *et al.*,2019) city who show that

In table ( 4-10) showed that concentration of TLR2 in patients with breast tumors with mean 9.953g was increase significantly compare with control group with mean 6.774, this study was agree with study (EL-kharashy *et al*.,2021) who showed that Ag This study was agree with study (Abdulabbas and Shani,2022) in Basra who showed that the serum levels

This study was disagree with study (Al-Ammiri and, Al-Derzi ,2013) who showed that

**Table (4-11) concentration of IL1α, Ilβ and TLR2 in patients group and healthy**

| Parameters | Concentration pg/ml | | | P value |
|---|---|---|---|---|
| | Mg ±SD | | | |
| | Breast cancer | Benign tumor | Healthy | |
| **IL1 α** | 0.891 ± .288 ab | 1.208 ±0.589 b | 0.616 ± 0.239 a | 0.001** |
| **IL1β** | 4.630 ± 2.434 a | 4.270 ± 2.204 a | 3.640 ± 1.996 a | 0.5 |
| **TLR2** | 11.826 ± 4.305 b | 8.967 ± 4.556 ab | 6.773 ± 3.855 a | 0.03* |

In table (4-11) show that concentration of IL1α in malignant breast with mean was 0.891 , in benign breast tumors the mean was 1.208g but in healthy with mean 0.616 ,

This study show that the mean of TLR2 was increase significantly in malignant breast tumors this study was agree with study ( Al- ammiri and Al-Derzi ,2013 ) who show that :

In this study the mean of TLR2g in benign breast tumors was 8.967 no significant increase these study was disagree with study (Al-g ammiri and Al-Derzig ,2013)

| Parameters / Age/years | concentration pg/ml ( M±SD) | | | P-g value |
|---|---|---|---|---|
| | 14-29 | 30-45 | 46-66 | |
| **TLR2 systemic** | 9.632±4.472 | 11.9131±3.040 | 7.710±5.869 | 0.1 |
| **TLR2tissue** | 0.184±0.084 | 0.251±0.175 | 0.160±0.114 | 0.3 |
| **IL1 beta systemic** | 4.895±2.710 | 6.254±4.010 | 6.433±4.960 | 0.6 |
| **IL1 beta tissue** | 20.833±12.215 | 20.333±17.643 | 22.619±9.567 | 0.9 |

**(4-12) Systemic and Local of TLR2 , IL1 beta and IL1 alpha in Patients with Breast Tumors According to Age Groups**

| | | | | |
|---|---|---|---|---|
| **IL1 alpha systemic** | 1.268±0.756 | 1.064±0.485 | 1.171±0.463 | 0.7 |
| **IL1 alpha tissue** | 1.679±0.780 | 0.626±0.338 | 1.444±0.825 | 0.002** |

In table (4-12)g the result of this study 0.626g but in third age group with mean 1.444 the result shows significant different in the concentration of IL1g α between age group, but in serum the mean of IL α in first age group was 1.268 , in second group the mean was 1.064 and in third age group was1.171 the result no significant different between age groups, IL1 β in patients serum in first age group was 4.895 , in second group the mean was 6.254andg in third age group was 6.433 the result no significant different between age groups , IL1 β in patients tissue in first age group was 20.833, in second group the mean was 20.333 and in third age group was 22.619 the result no significant different between age groups ,

TLR2g in patients serum in first age group was 9.632 , in second group the mean was 11.9131andg in third age group was 7.710the result no significant different between age groups , TLR2 in patients tissue in first age group was 0.184, in second group the mean was 0.251andg in third age group was 0.160 the result no significant different between age groups this agree with study (Abdulabbas and Shani,2022g ) who showed that

The present study showed that no significant different in concentration of IL1 beta in patient serum between age group this study was agree with study (Lafrenie *et al*.,2023) who showed that no significant between age group and IL1 beta concentration

**Table (4-13) concentration of IL1α ,g IL1 β and TLR2 between patients before and after chemotherapy**

| Parameters | Concentration pg/ml M±SD | | P_ value |
|---|---|---|---|
| | Before chemotherapy | After chemotherapy | |
| **IL1α** | 1.301±0.913 | 0.619±0.323 | 0.03* |
| **IL1β** | 5.598±3.550 | 17.593±16.354 | 0.001* |
| **TLR2** | 9.953±4.606 | 7.774±2.674 | 0.2 |

*(p≤0.05) is considered significant

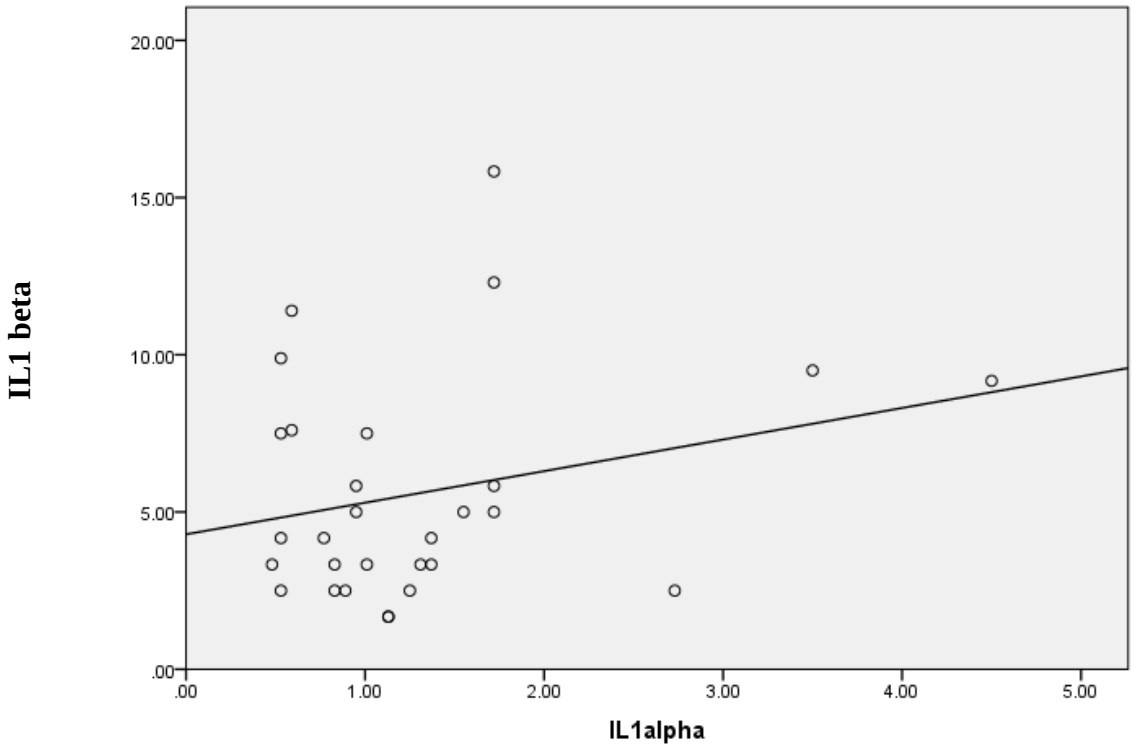
In table (4-13) showed that the of IL1 α in patients without chemotherapy was 1.301 but mean of IL1 α with chemotherapy was 0.619 were significant different between , the mean IL1 β patients without chemotherapy was 5.598g but mean of IL β with chemotherapy was 17.593, but the mean of TLR2 without chemotherapy was 9.953 but mean of TLR2 after chemotherapy was 7.774 .this study showed that increase significantly of IL1α and IL1β without and with chemotherapy this study was agree with study (Tsavaris *et al*.,2022) who showed that

Also disagree with study (Felix *et al*.,2018 )

**Table (4-14)Comparison between concentration of TLR2 , IL1 alpha and IL1 beta between patients in blood and tissue patients**

| Parameters | M±SD | | P- value |
|---|---|---|---|
| | Blood | Tissue | |
| **TLR2** | 9.953±4.606 | 0.209±0.143 | 0.000*** |
| **IL1 beta** | 5.599±3.550 | 21.000±14.356 | 0.001** |
| **IL1 alpha** | 1.304±0.913 | 1.086±0.818 | o.4 |

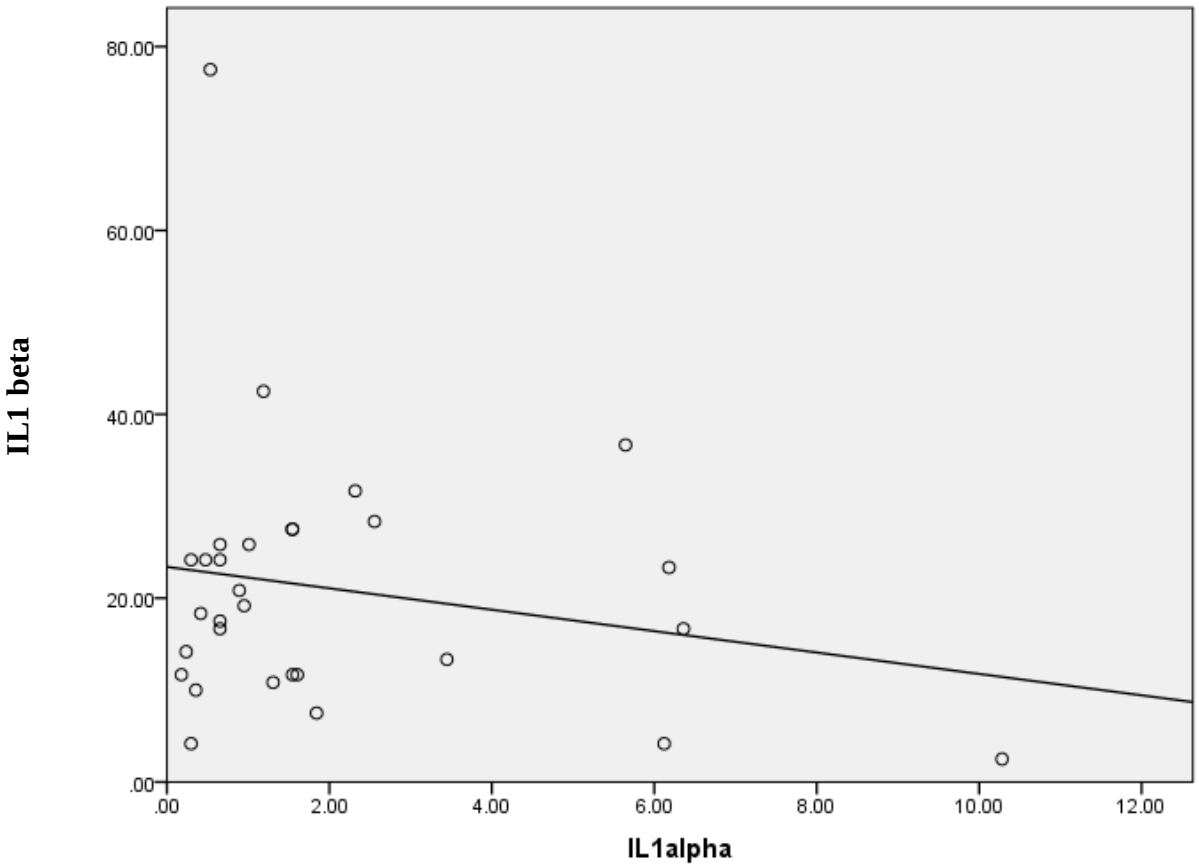
*(p≤ 0.05) is considered significant




P=0.1

R=0.3

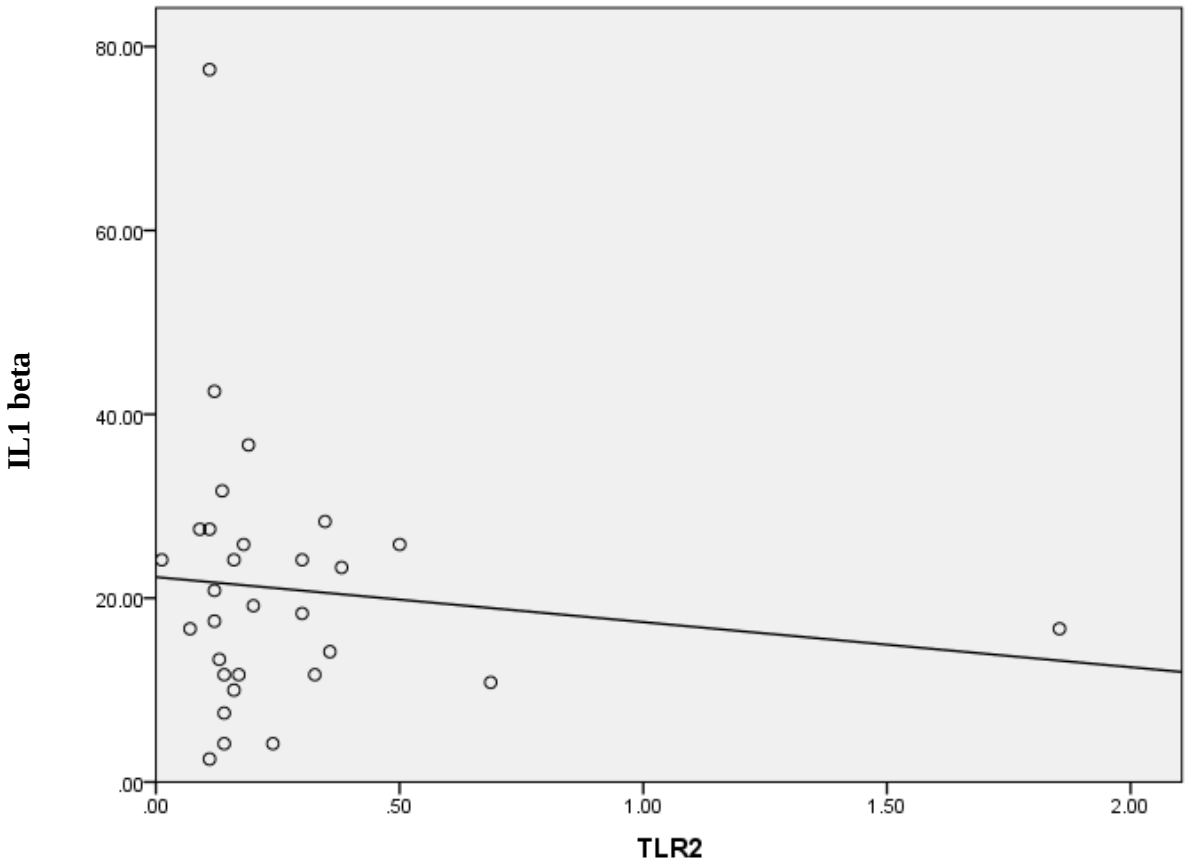
**Figure (4-3)Correlation between IL1 BETA and IL1 alpha blood**




P= 0.3

R=-0.2

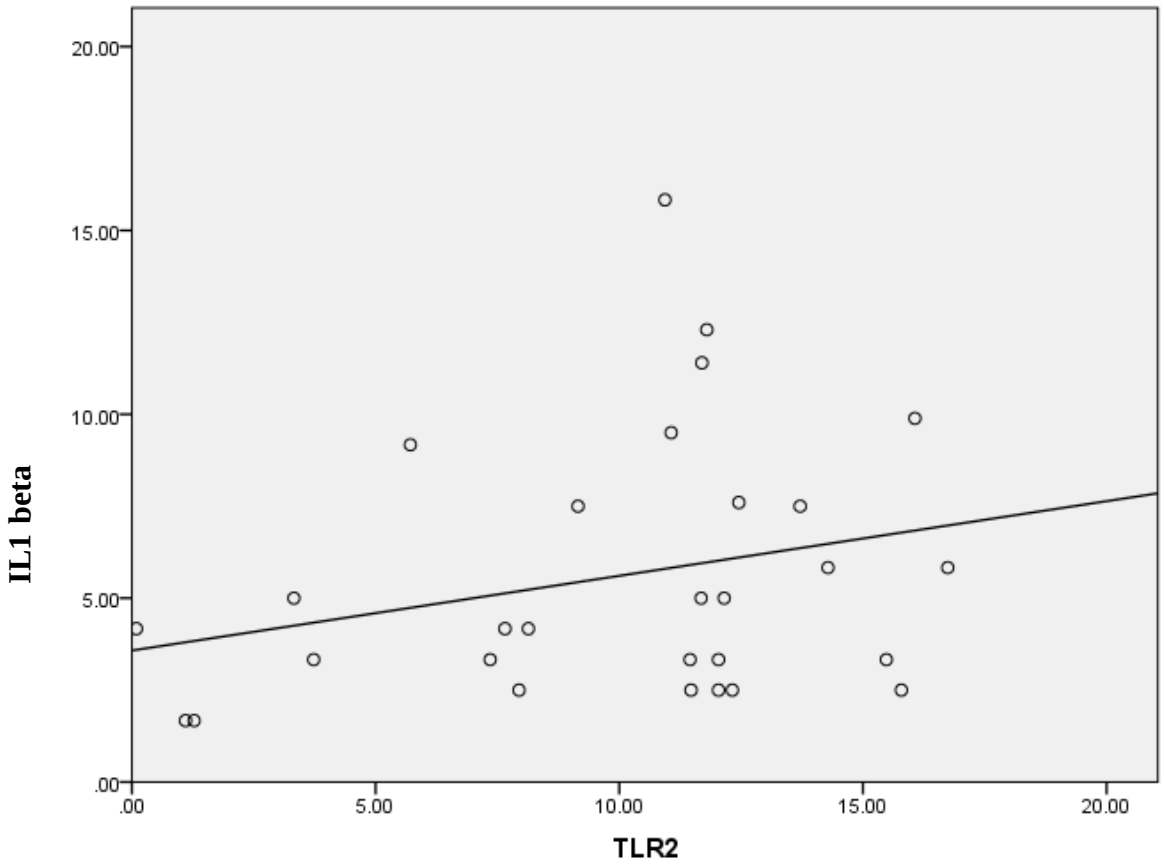
**Figure (4-4) Correlation between IL1 beta and IL1 alpha mucosal**




P= 0.5

R=-0.1

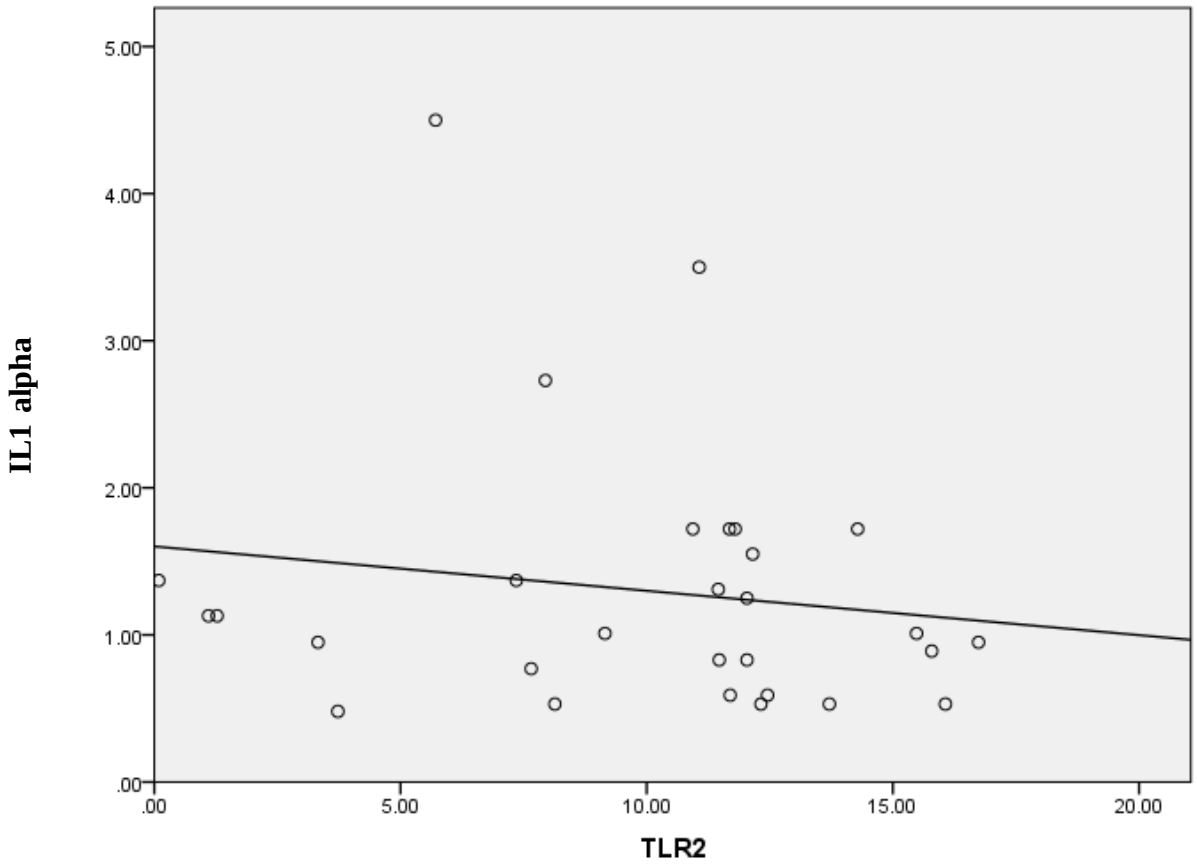
**Figure (4-5) Correlation between TLR2 and IL1 BETA in tissue**




P= 0.1

R=0.3

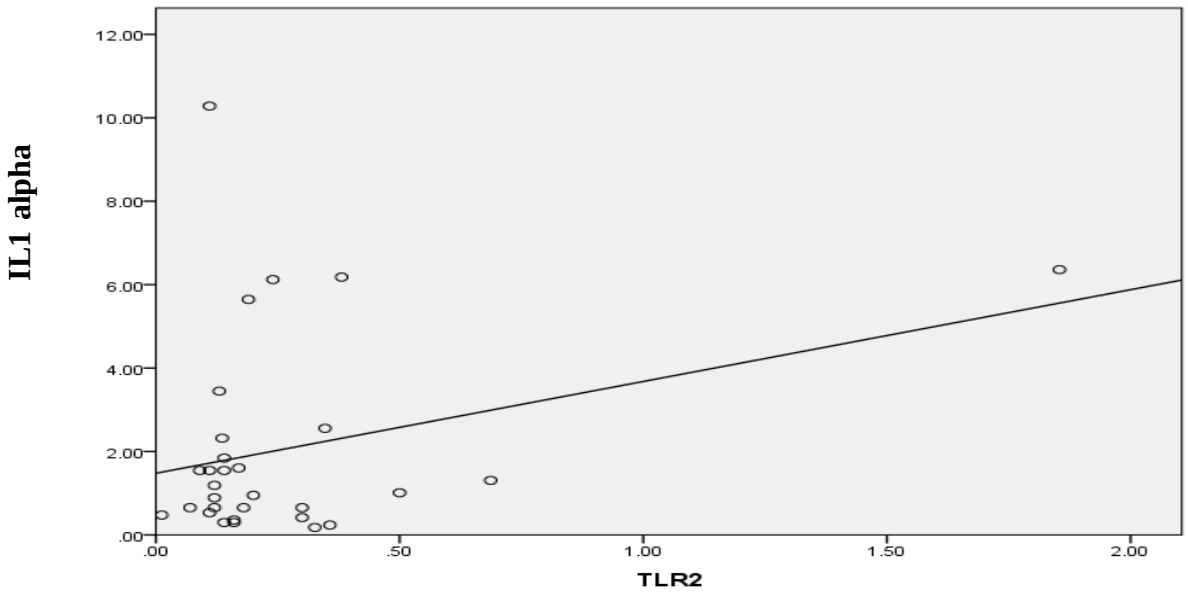
**Figure (4-6) Correlation between TLR2 and IL1 beta in blood**




P=0.4

R=-0.2

**Figure (4-7) Correlation between TLR2 and IL1 alpha in blood**




P= 0.1

R=0.3

**Figure (4-8) Correlation between TLR2 and IL1 alpha in tissueg**

## 4-5 Molecular study of breast tumors

### 4-5-1 IL-1 alpha -889 C>T promoter primer

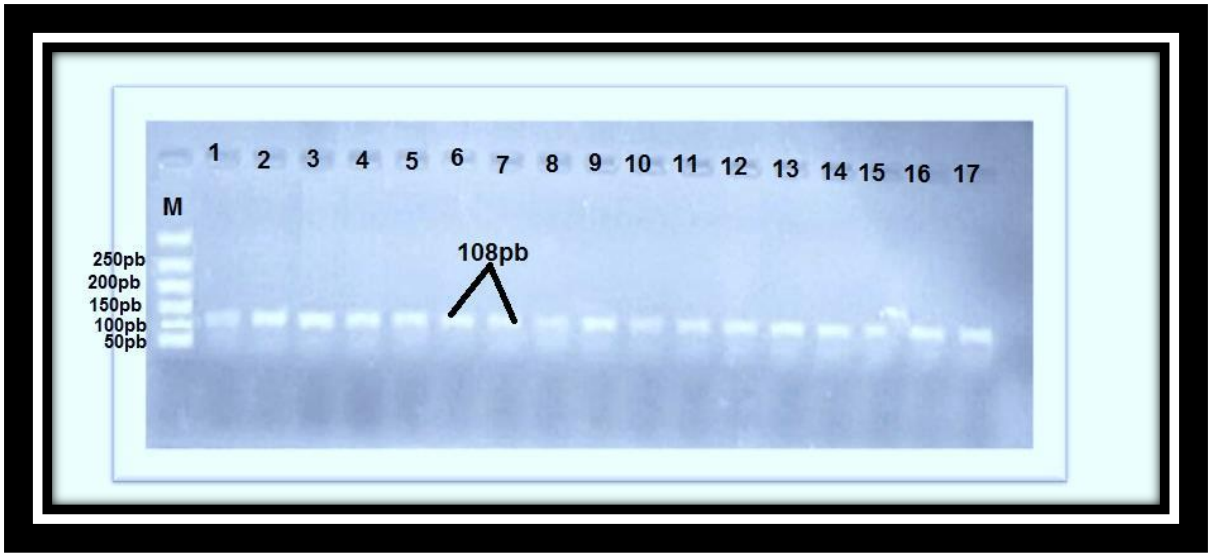



**Figure (4-9) Electrophoreses pattern of PCR product of IL-1 alpha -889 C>T in blood, M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 53.9**

PCR product of IL-1 alpha -889 C>T gene was amplified by using specific primer . the PCR product (band ) of IL-1 alpha -889gC>T gene was 108 –bp in tissue patients figure (4-10) .

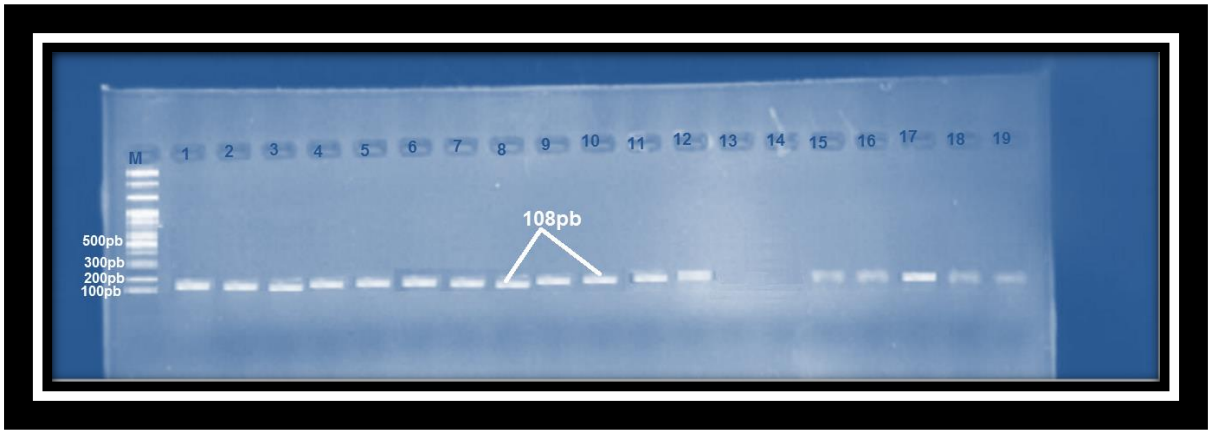



**Figure (4-10) Electrophoreses pattern of PCR product of IL-1 alpha -889 C>T in tissue , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 53.9**

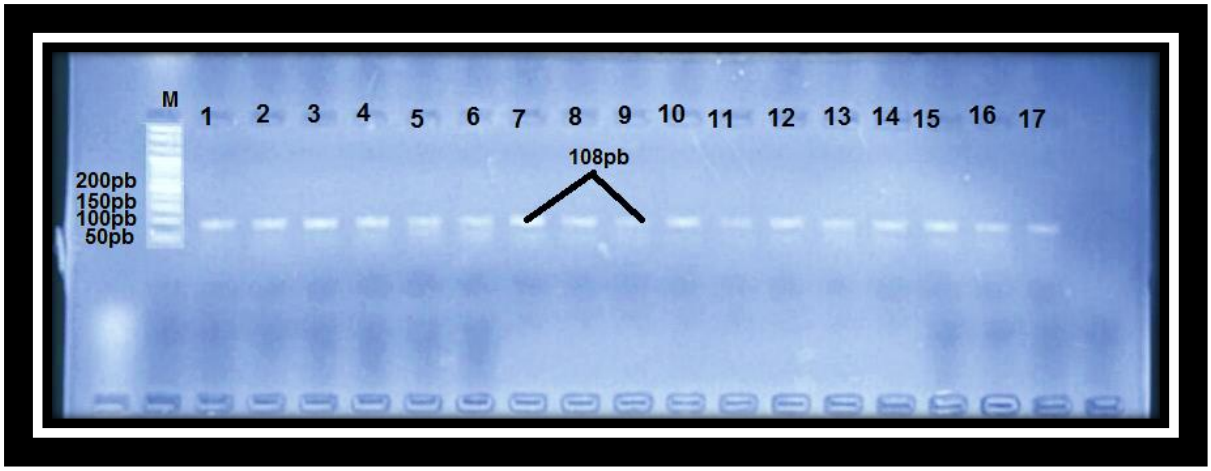



**Figure (4-11) Electrophoreses pattern of PCR product of IL-1 alpha -889 C>T in blood of chemotherapy , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 53.9**

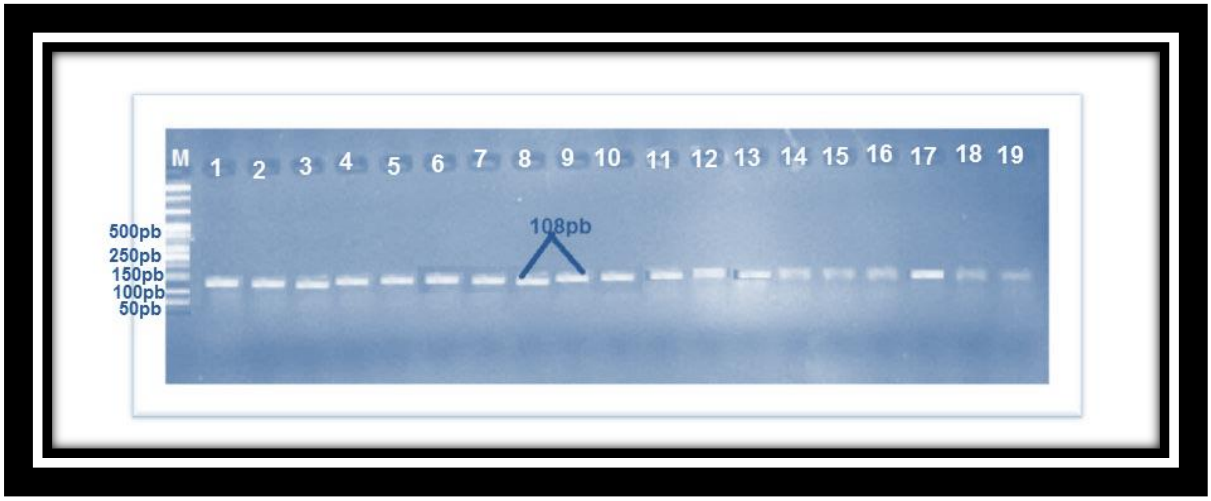


**Figure (4-12) Electrophoreses pattern of PCR product of IL-1 alpha -889 C>T blood control , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 53.9**

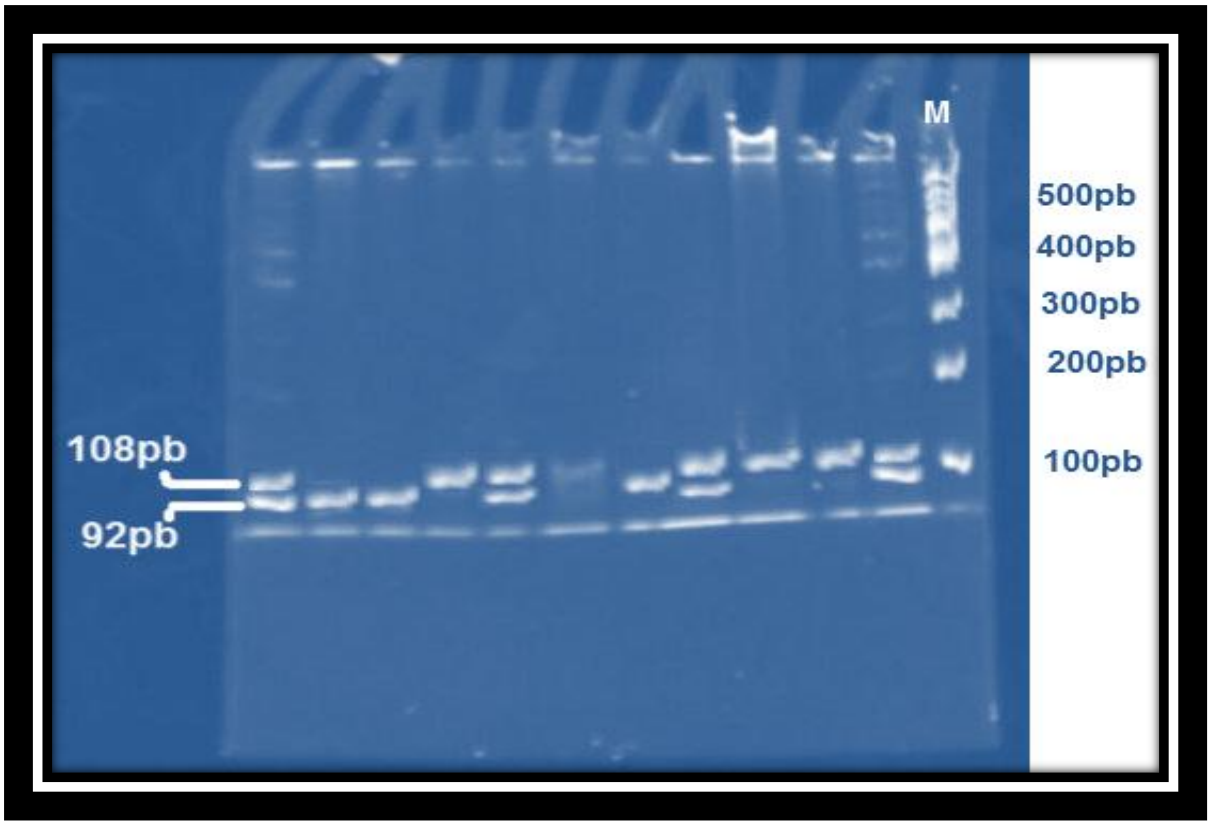



**figure ( 4-13 ) electrophorases patteren of IL-1 alpha -889 C>T gene PCR-RFLP by PAGE gel for PCR product (108pb) with restriction enzyme *NocI* . M : DNA ladder . lane (5,9,10) homozygote TT genotype 92pb , lane (2,3,8) homozygote (CC) genotype (108pb) lane (1,4,6,7,11) heterozygote (CT) genotype (108pb and 92 pb)**

%)and 25(25%) in blood patients ,where it were 17(34%), 20(40%) and 13(26%) in healthy groups table (4-15) .the P-value of the each genotypes frequencies of IL-1 alpha -889 C>T gene were nosignificant 0.57 and 0.47 for CT and TT respectively , patients with genotype CT were

affected by breast tumors approximately one time comparsion with patients having genotype TT (odd ratio = 1.28 and 0.72 .

**Table (4-15)genotype frequency of IL-1 alphas -889 C>T gene polymorphism with allele frequency in healthy control and blood breast tumors patients**

| **Genotype IL-1g alpha -889 C>T** | **Blood patients** | **Healthy (control )** | **P- value** | **Odd ratio** |
|---|---|---|---|---|
| **CC** | 25(25%) | 13(26%) | | |
| **CT** | 30(30% | 20(40% | 0.57 | **1.28(0.53- 3.08)** |
| **TT** | 45(45%) | 17(34%) | 0.47 | **0.72( 0.30-1.73)** |
| **Total number** | 100 | 50 | | |
| **Allele frequency** | | | | |
| **C** | 80(0.4) | 46(0.54) | | |
| **T** | 120(0.6) | 54(0.46) | 0.32 | **0.78( 0.48-1.27)** |

In tissue patient C>T gene polymorphism where it were 15(21%), 30(30%) and 25(36%) in the breast tumors patients , table (4-16) . CT patients with genotype CT were affected by breast tumors approximately one time comparison with patients having genotype TT (odd ratio = 1.00 and 0.33) .

**Table (4-16)genotype frequency of gene polymorphism with allele frequency in blood patients and tissue breast tumors patients**

| Genotype IL-1 alpha -889 C>T | blood patients | Tissue patients | P- value | Odd ratio |
|---|---|---|---|---|
| **CC** | 25(25%) | 25(36%) | | |
| **CT** | 30(30%) | 30(30%) | 1.00 | 1.00(047-2.11) |
| **TT** | 45(45%) | 15(21%) | 0.007* | 0.33(0.14- 0.74) |
| **Total number** | 100 | 70 | | |
| **Allele frequency** | | | | |
| **C** | 80(0.4) | 80(0.57) | | |
| **T** | 120(0.6) | 60(0.4) | 0.002* | 0.50(0.32- 0.77) |

in chemotherapy patients table (4-17)in the breast tumors patients the P-value of the genotypes frequencies of IL-1 alpha -889 C>T gene were significantly 0.007 for TT ($p<0.05$) and no significant for CC and CT patients with genotype TT were affected by breast tumors approximately one time comparison with patients having genotype CT (odd ratio = 1.50g and 0.91)

**Table (4-17)genotype frequency of gene polymorphism with allele frequency in healthy control and blood breast tumors patients**

| Genotype IL-1 | Chemotherapy | Healthy | P value | OR |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| **alpha -889 C>T** | | | | **CL 95%** |
| **CC** | 10(33%) | 16(32%) | | |
| **CT** | 15(50%) | 22(44%) | 0.86 | 0.91( 0.32-2.56) |
| **TT** | 5(17%) | 12(24%) | 0.54 | 1.50( 0.40-5.55) |
| **Total** | 30 | 50 | | |
| **Alleles frequency** | | | | |
| **C** | 35(0.58) | 54 | | |
| **T** | 25(0.41) | 46 | 0.59 | 1.19( 0.62- 2.27) |

In blood benign patients table (4-18)in the breast tumors patients the P-value of the genotypes frequencies of IL-1 alpha -889 C>T gene were no significantly where patients with TT more affected by breast tumors comparison with patients having genotype CT(Odd ratio 0.90 , 0.70)

| **Genotype**<br>**IL-1 alpha -889 C>T** | **Malignant**<br>**Blood** | **Benign**<br>**Blood** | **P value** | **OR**<br>**CL 95%** |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| CC | 12(29%) | 20(34%) | | |
| CT | 22(52%) | 26 (45%) | 0.46 | 0.70(0.28-1.76) |
| TT | 8 (19%) | 12 (21%) | 0.85 | 0.90( 0.28-2.83) |
| Total | 42 | 58 | | |
| Alleles | | | | |
| C | 46(0.55) | 66(0.55) | | |
| T | 38(0.45) | 50(0.45) | 0.76 | 0.91( 0.52-1.61) |

**Table (4-18)genotype frequency of gene polymorphism with allele frequency in Malignant and Benign blood breast tumors patients**

In tissue benign patients table (4-19)in the breast tumors patients the P-value of the genotypes frequencies of IL-1 alpha -889 C>T gene were no significantly ,whereas patients with CT more affected by breast tumors comparison with patients having genotype TT(Odd ratio 1.16, 0.98).

**Table (4-19)genotype frequency of gene polymorphism with allele frequency in Malignant and Benign blood breast tumors patients**

| **Genotype IL-1 alpha -889 C>T** | **Malignant Tisuue** | **Benign tissue** | **P value** | **OR CL 95%** |
|---|---|---|---|---|
| CC | 12(50%) | 22(48%) | | |
| CT | 7(29%) | 15(19%) | 0.78 | **1.16( 0.73-3.65)** |

| | | | | |
|---|---|---|---|---|
| TT | 5(21%) | 9(33%) | 0.97 | **0.98( 0.26-3.60)** |
| Total | 24 | 46 | | |
| **Alleles frequency** | | | | |
| C | 31(0.66) | 59(0.64) | | |
| T | 17(0.35) | 33(0.36) | 0.95 | **1.02( 0.49-2.11)** |

### 4-5-2 IL-1 beta C31 T

#### 4-5-2-1 IL-1 beta C31 T genotyping PCR

PCR product of IL-1 beta C31 T gene was amplified by using specific primer . the PCR product (band )


**Figure (4-14) Electrophoreses pattern of PCR product of IL-1 beta C31 T blood patients and control, M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 54.8**

PCR product of IL-1 beta C31 T gene was amplified by using specific primer . the PCR product (band ) of IL-1 beta C31 T gene was 240 –bp in tissue patients figure (4-15) .


**Figure (4-15) Electrophoreses pattern of PCR product of IL-1 beta C31 T tissue patients , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 54.8**

PCR product of IL-1 beta C31 T gene was amplified by using specific primer . the PCR product (band ) IL-1 beta C31 T gene was 240 –bp in chemotherapy patients figure (4-16) .

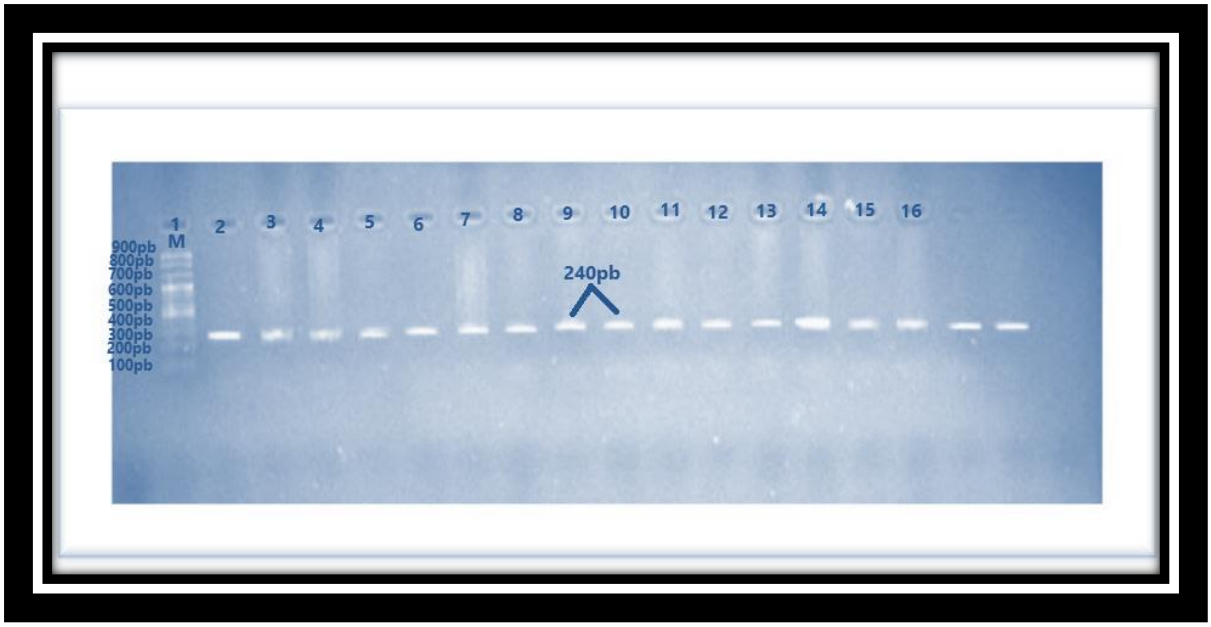
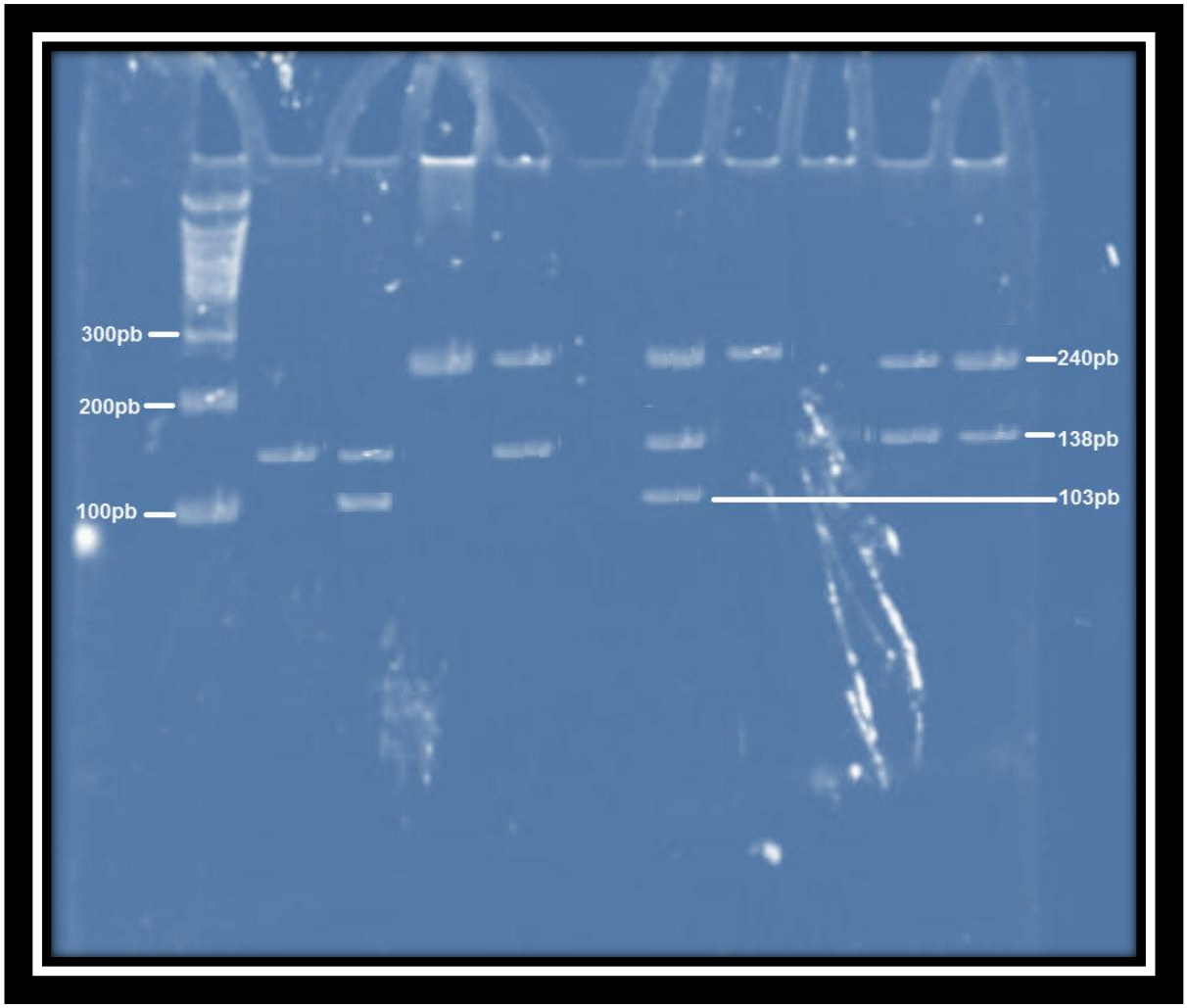



**Figure (4-16) Electrophoreses pattern of PCR product of IL-1 beta C31 T blood chemotherapy , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 54.8**




,where it were 17(34%) , 20(40%)and 13(26%)in healthy groups table (4-20) . by breast tumors approximately comparison with patients having genotype CT (odd ratio = 0.93 and 0.59 ).in present study CC and CT not related with breast cancer this agree with study in turkey ( Eras *et al*.,2019),who showed that CT heterozygote genotype was not related with breast cancer ,

**Tablg (4-20)Genotype of IL-1 beta C31 T gene polymorphism with allele frequency in patients' blood and healthy**

| Genotype IL-1 beta C31 T | Patients (blood) | Healthy | P value | OR<br>CL 95% |
|---|---|---|---|---|
| CC | 20(20%) | 13(26%) | | |

| | | | | |
|---|---|---|---|---|
| **CT** | 52(52%) | 20(40%) | 0.23 | 0.59( 0.24-1.41) |
| **TT** | 28(28%) | 17(34%) | 0.88 | 0.93( 0.37-2.34) |
| **Total** | 100 | 50 | | |
| **Alleles frequency** | | | | |
| **C** | 92(0.46) | 46(0.46) | | |
| **T** | 108(0.54) | 54(0.54) | 1.00 | 1.00( 0.61-1.61) |

In tissue patient table (4-21) .the P-value of the genotypes frequencies of IL-1 beta C31 T gene were no significantly for TT ($p<0.05$) and no significant for CC and CT patients with genotype CT were affected by breast tumors approximately one time comparison with patients having genotype TT (odd ratio = 0.62 and 0.58) .

**Table (4-21)Genotype of IL-1 beta C31 T gene polymorphism with allele frequency in patients' blood and tissue**

| Genotype IL-1 beta C31 T | Patients (Blood ) | Patients (tissue ) | P value | OR CL 95% |
|---|---|---|---|---|
| **CC** | 20(20%) | 7(23%) | | |
| **CT** | 52(52%) | 14(47%) | 0.62 | 0.76( 0.27-2.18) |
| **TT** | 28(28%) | 7(23%) | 0.58 | 0.71( 0.21-2.35) |
| **Total** | 100 | 28 | | |
| **Alleles frequency** | | | | |
| C | 92(0.46) | 28(0.56) | | |
| T | 108(54) | 28(0.44) | 0.77 | 0.85( 0.28- 2.51) |

In chemotherapy beta C31 T gene were no significantly for TT and no significant for CC and CT patients with genotype TT were affected by breast tumors approximately one time comparison with patients having genotype CT (odd ratio = 1.01 and 0.76) .

**Table (4-22)Genotype of IL-1 beta C31 T gene polymorphism with allele frequency in patients with chemotherapy and healthy**

| **Genotype IL-1 beta C31 T** | **(Chemotherapy )** | **Healthy** | **P value** | **OR CL 95%** |
|---|---|---|---|---|
| **CC** | 7(23%) | 13(26%) | | |
| **CT** | 14(47%) | 20(40%) | 0.65 | 0.76( 0.24-2.41) |
| **TT** | 9(30%) | 17(34%) | 0.97 | 1.01( 0.29-3.45) |
| **Total** | | | | |
| **Frequency Alleles** | | | | |
| **C** | 28(0.47) | 46(0.46) | | |
| **T** | 32(0.53) | 54(0.54) | 0.93 | 1.02(0.54-1.95) |

In blood benign patients (32%) , 12(48%) and 5(20%)table (4-23)in the breast tumors patients the P-value of the genotypes frequencies of IL-1 beta C31 T gene were no significantly where's patients with TT , CT

more affected by breast tumors comparison with patients having genotype CT(Odd ratio 0.31 ,g 0.31).

**Table (4-23)genotype frequency of IL-1 beta C31 T gene polymorphism with allele frequency in Malignant and Benign blood breast tumors patients**

| Genotype IL-1 beta C31 T | Malignant Blood | Benign Blood | P value | OR CL 95% |
|---|---|---|---|---|
| **CC** | 5(20%) | 20(45%) | | |
| **CT** | 12(48%) | 15(33%) | 0.06 | 0.31( 0.09-1.07) |
| **TT** | 8(32%) | 10(22%) | 0.08 | 0.31(0.08-1.20) |
| **Total** | 25 | 45 | | |
| **Alleles frequency** | | | | |
| **C** | 22(0.41) | 55(0.54) | | |
| **T** | 28(0.59) | 35(0.38) | 0.05* | 0.50( 0.24-1.008) |

In tissue benign patients for TT genotype ($p<0.05$) but not significant for CT and CC respectively ,whereas patients with CT more affected by breast tumors comparison with patients having genotype TT(Odd ratio 0.38, 0.26).

**Table (4-24)genotype frequency of IL-1 beta C31 T gene polymorphism with allele frequency in Malignant and Benign blood breast tumors patients**

| Genotype IL-1 beta C31 T | Malignant Tissue | Benign Tissue | P value | ORCL 95% |
|---|---|---|---|---|
| **CC** | 8(17%) | 18(37%) | **g** | |
| **CT** | 23(48%) | 20(42%) | 0.06 | **0.38( 0.13-1.07)** |
| **TT** | 17(35%) | 10(21%) | 0.019* | **0.26( 0.08-0.81)** |
| **Alleles frequency** | | | | |
| **C** | 29(0.44) | 56(0.61) | | |
| **T** | 57(0.56) | 40(0.38) | <0.001* | **0.36(0.19-0.66)** |

,

### 4-5-3 Toll like receptor 2 (TLR2)

### 4-5-3-1TLR2 Asp 299 Gly genotyping PCR

PCR product of TLR2 Asp 299 Gly gene was amplified by using specific primer . the PCR product (band ) of TLR2 Asp 299 Gly gene was 286 – bp in patients and control blood figure (4-18) .

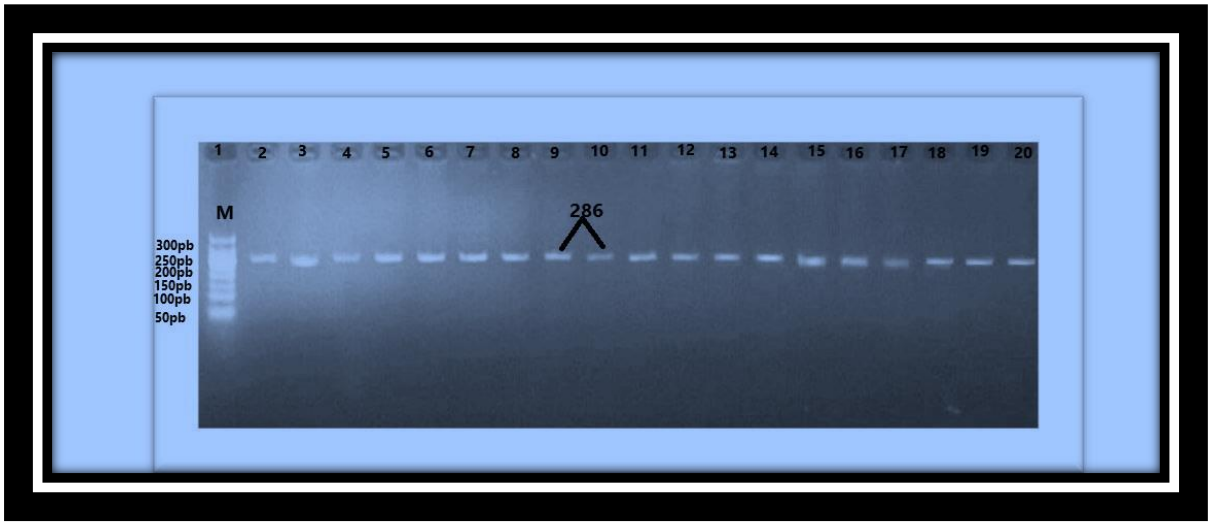


**Figure (4-18) Electrophoreses pattern of PCR product of TLR2 Asp 299 Gly patients and control blood , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 62**

PCR product of TLR2 Asp 299 Gly gene was amplified by using specific primer . the PCR product (band ) TLR2 Asp 299 Gly gene was 286 –bp in chemotherapy patients figure (4-19) .

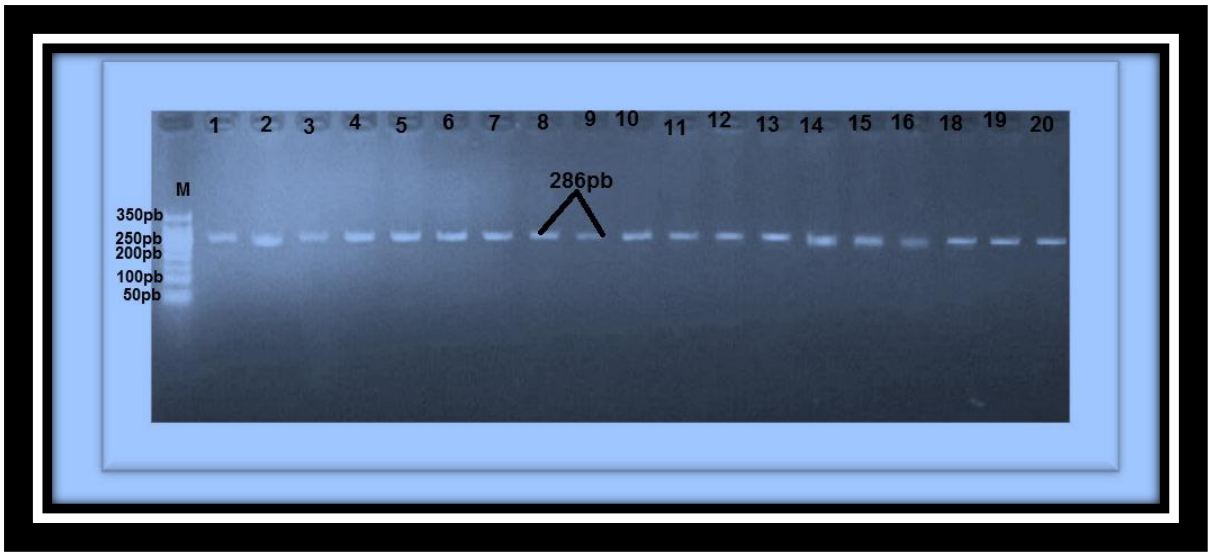



**Figure (4-19) Electrophoreses pattern of PCR product of TLR2 Asp 299 Gly in blood chemotherapy and tissue , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 62**

### 4-5-3-2 DNA sequencing of TLR2 Asp 299 Gly

To prove the result of TLR2 Asp 299 Gly, sequencing were ,the result detection , insertion occur in Guanine , cytosine , thymine and adenine figure (4-20 ).

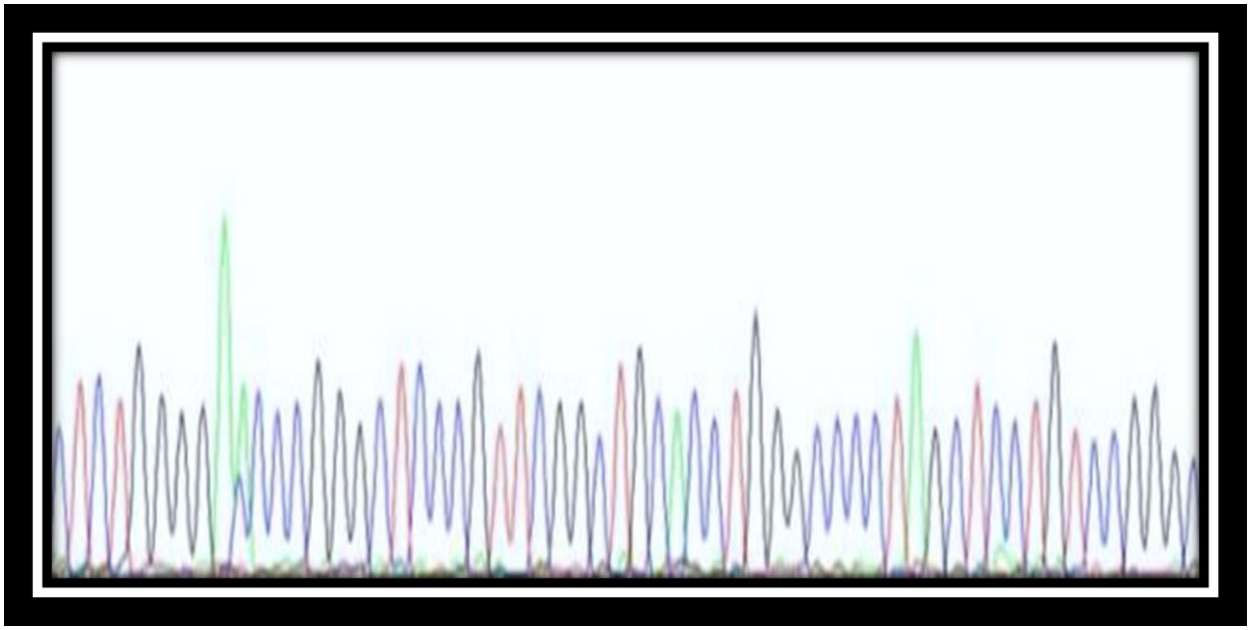


**Figure (4-20) DNA sequencing of TLR2 Asp 299 Gly**

**Table (4-25) number and percentage for nucleotide deletion between patients and control**

| Nucleotide | Deletion | | | |
|---|---|---|---|---|
| | **Number / percentage** | | | |
| | **Patients' blood** | **Healthy (control)** | | |
| **G** | **42(49%)** | **20(43%)** | | |
| **C** | **33(39%)** | **19(41%)** | | |
| **A** | **6(7%)** | **4(9%)** | | |
| **T** | **4(5%)** | **3(7%)** | | |

**Table (4-26) number and percentage for nucleotide deletion between patients' blood and tissue patients**

| Nucleotide | Deletion | | | |
|---|---|---|---|---|
| | **Number / percentage** | | | |
| | **Patients' blood** | **Patients tissue** | | |
| **G** | **42(49%)** | **36(45%)** | | |
| **C** | **33(39%)** | **34(42%)** | | |
| **A** | **6(7%)** | **8(10%)** | | |
| **T** | **4(5%)** | **2(3%)** | | |

**Tableg (4-27)g numberg andg percentageg forg nucleotideg deletiong betweeng patients'g bloodg andg tissueg patients**

| Nucleotide | Deletion | | | |
|---|---|---|---|---|
| | **Numberg /g percentage** | | | |
| | **Chemotherapyg patients** | **Healthyg (control)** | | |
| **G** | **17(55%)** | **20(43%)** | | |
| **C** | **10(32%)** | **19(41%)** | | |
| **A** | **3(10%)** | **4(9%)** | | |
| **T** | **1(3%)** | **3(7%)** | | |

**g**

**Table (4-28) number and percentage for nucleotide deletion between patients and control**

| Nucleotide | Insertion | | | |
|---|---|---|---|---|
| | **Number / percentage** | | | |
| | **Patients' blood** | **Healthy (control)** | | |
| **G** | **12(27%)** | **6(34%)** | | |
| **C** | **11(24%)** | **4(22%)** | | |
| **A** | **6(13%)** | **4(22%)** | | |
| **T** | **16(36%)** | **4(22%)** | | |

**Table (4-29) number and percentage for nucleotide deletion between patients and control**

| Nucleotide | Insertion | | | |
|---|---|---|---|---|
| | **Number / percentage** | | | |
| | **Patients' blood** | **Patients tissue** | | |
| **G** | **12(27%)** | **5(50%)** | | |
| **C** | **11(24%)** | **2(20%)** | | |
| **A** | **6(13%)** | **1(10%)** | | |
| **T** | **16(36%)** | **2(20%)** | | |

**Table (4-30) number and percentage for nucleotide deletion between patients and control**

| Nucleotide | Insertion | | | |
|---|---|---|---|---|
| | **Number / percentage** | | | |
| | **Chemotherapy patients** | **Healthy (control)** | | |
| **G** | **1(8%)** | **6(34%)** | | |
| **C** | **4(31%)** | **4(22%)** | | |
| **A** | **2(15%)** | **4(22%)** | | |
| **T** | **6(46%)** | **4(22%)** | | |

**4**

**4-5-4 -1 TLR4 3725 G / C genotyping PCR**

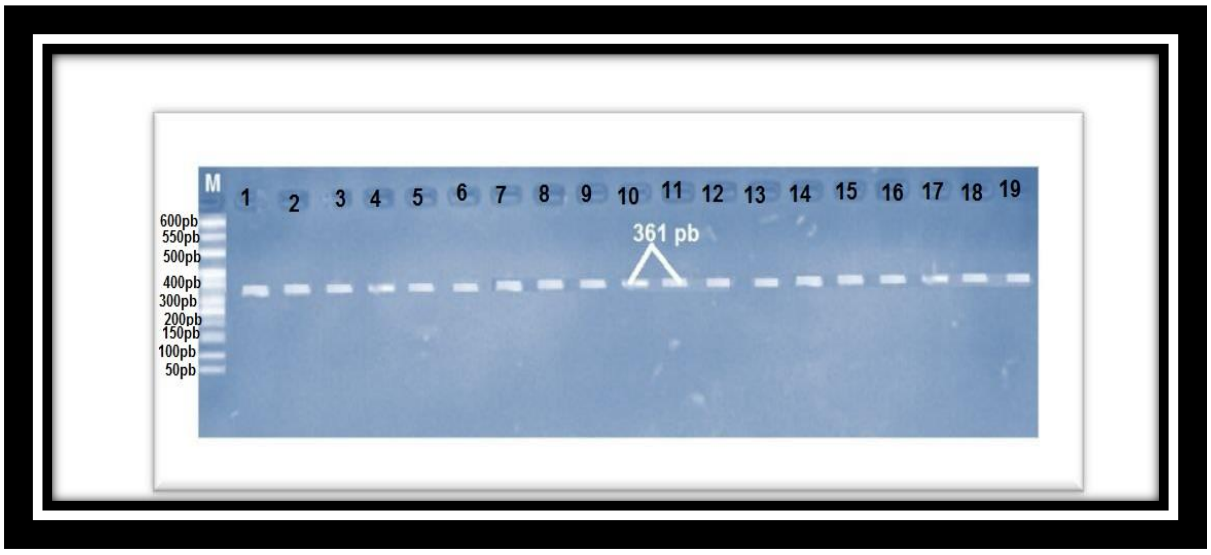


**Figure (4-21) Electrophoreses pattern of PCR product of TLR4 3725 G / C blood patients and control , M : molecular DNA ladder , 1-19 PCR product , the optimum annealing temperature was 57.5**

g PCR product of TLR4 3725 G / C gene was amplified by using specific primer . the PCR product (band ) of TLR4 3725 G /g Cg gene was 361 – bp in tissue patients figure (4-22) .

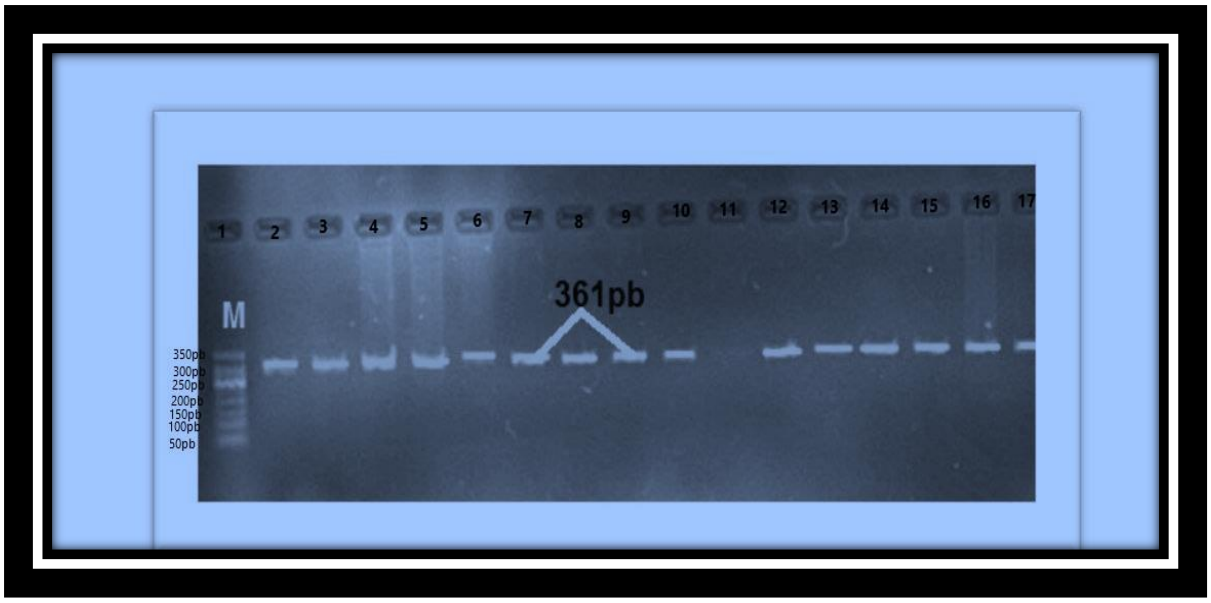



**Figure (4-22) Electrophoreses pattern of PCR product of TLR4 3725 G / C in tissue patients , M : molecular DNA ladder , 1-17 PCR product , the optimum annealing temperature was 57.5 in tissue patients**

PCR product of TLR4 3725 G / C gene was amplified by using specific primer . the PCR product (band ) TLR4 3725 G / C gene was 361 –bp in chemotherapy patients figure (4-23) .

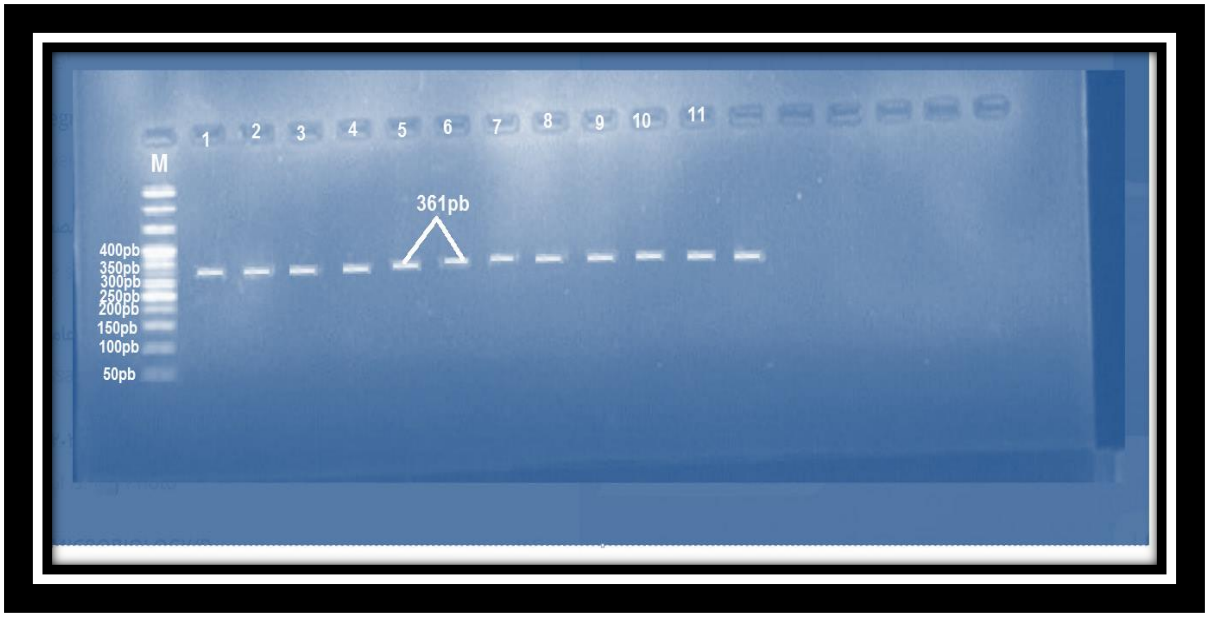



**Figure (4-23)g Electrophoreses pattern of PCR product of TLR4 3725 G / C in blood chemotherapy , M : molecular DNA ladder , 1-17g PCR product , the optimum annealing temperature was 57.5**

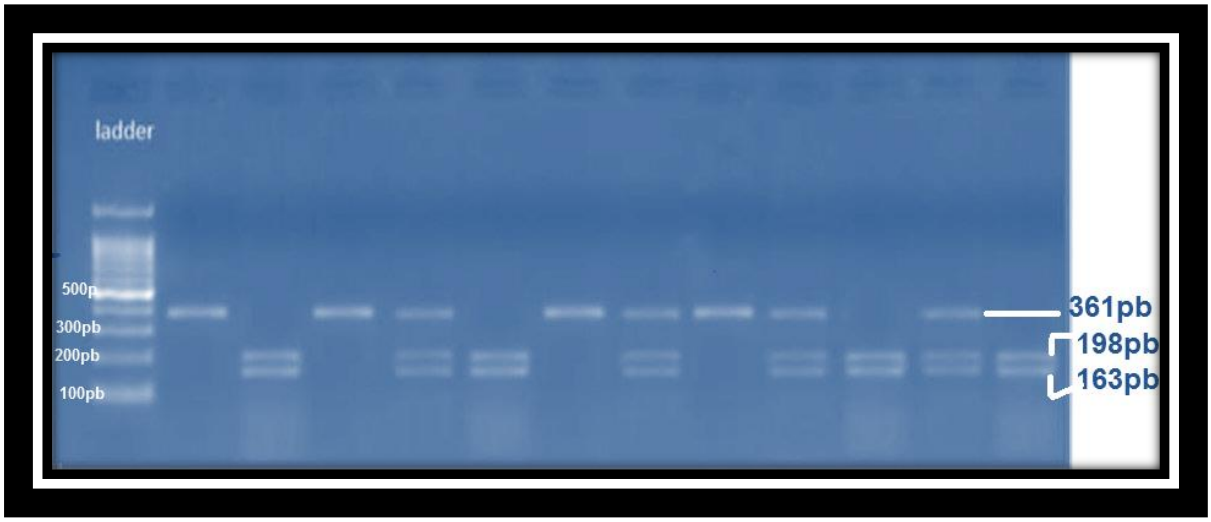



**Figure (g 4-24g )g ) electrophoresis pattern of TLR4 3725 G / C gene**

g

g Genotype, 8(38%) and 1(5%)g in healthy groups table (6) .the P-value of the each genotypes frequencies of TLR4 3725 G / C gene were no significant 0.47 and 0.06 for GC and CC respectively , patients with

genotype GC were affected by breast tumors approximately one time comparison with patients having genotype CC (odd ratio = 1.44 and 0.7) .

**Table (4-31)genotype frequency ofTLR4 3725 G / C gene polymorphism with allele frequency in healthy control and blood breast tumors patients**

| Genotype TLR4 3725 G / C | Blood patients | Healthy | P value | OR CL 95% |
|---|---|---|---|---|
| **GG** | 54(51%) | 12(57%) | | |
| **GC** | 25(24%) | 8(38%) | 0.47 | **1.44(0.52-3.96)** |
| **CC** | 26(25%) | 1(5%) | 0.06 | **0.17( 0.02-1.40)** |
| **Total** | 95 | 21 | | |
| **Alleles frequency** | | | | |
| **G** | 133(0.7) | 32(0.76) | | |
| **C** | 77(0.41) | 10g (0.24) | 0.11 | **0.54( 0.25- 1.15)** |

%) , 15(21%) and 23(33%) in tissue patients ,where it were 54(51%) , 25(24%) and 26(25%)in blood patients groups table (4-32)g .the P-value of the each genotypes frequencies of TLR4g 3725 G / C gene were no significant 0.97 and 0.26 for GC and CC respectively , patients with genotype CC were affected by breast tumors approximately one time comparison with patients having genotype GC (odd ratio = 1.49 and 1.01) .

**Table (4-32)genotype frequency of and tissue breast tumors patients**

| Genotype TLR4 3725 G / C | Blood patients | Tissue patients | P value | OR CL 95% |
|---|---|---|---|---|
| **GG** | 54(51%) | 32(46%) | | |
| **GC** | 25(24%) | 15(21%) | 0.97 | 1.01( 0.45-2.19) |
| **CC** | 26(25%) | 23(33%) | 0.26 | 1.49( 0.73-3.04) |
| **Total** | 95 | 70 | | |
| **Alleles frequency** | | | | |

| | | | | |
|---|---|---|---|---|
| **G** | 133(0.7) | 79(0.56) | | |
| **C** | 77(0.41) | 61(0.44) | 0.19 | 1.33( 0.86-2.06) |

In chemotherapy patients genotype g in healthy groups table (4-33) .the P-value of the each genotypes frequencies of TLR4 3725 G / C gene were no significant 0.49 and 0.20 for GC and CC respectively , patients with genotype GC were affected by breast tumors approximately one time comparison with patients having genotype CC(odd ratio = 0.66 and 0.25) .

**Table (4-33)genotype frequency of TLR4 3725 G / Cg gene polymorphism with allele frequency in healthy control and chemotherapy breast tumors patients**

| **Genotype TLR4 3725 G / C** | **Chemotherapy** | **Healthy** | **P value** | **OR CL 95%** |
|---|---|---|---|---|
| **GG** | 15(50%) | 12(57%) | | |
| **GC** | 10(33%) | 8(38%) | 0.49 | 0.66( 0.21-2.11) |
| **CC** | 5(17%) | 1(5%) | 0.20 | 0.25( 0.02-2.43) |
| **Total** | 25 | 21 | | |
| **Alleles frequency** | | | | |
| **G** | 40(0.8) | 32(0.76) | | |
| **C** | 20(0.4) | 10(0.24) | 0.29 | 0.62( 0.25- 1.52) |

In blood significantly with GC (0.02) , where's patients with GC more affected by breast tumors comparison with patients having genotype CC(Odd ratio 2.77 ,0.55).

**Table (4-34)genotype frequency of**

| Genotype TLR4 3725 G / C | Malignant blood | Benign blood | P value | OR CL 95% |
|---|---|---|---|---|
| **GG** | 20(48%) | 18(34%) | | |
| **GC** | 12(28%) | 30(57%) | 0.02* | 2.77(1.10-6.99) |
| **CC** | 10(24%) | 5(9%) | 0.35 | 0.55( 0.15-1.93) |
| **Total** | 42 | 53 | | |
| **Alleles frequency** | | | | |
| **G** | 52(0.62) | 66(0.62) | | |
| **C** | 32(0.38) | 40(0.38) | 0.96 | 0.85( 0.54-1.77) |

In Tissue benign were 16(67%) , 5(2%) and 3(2%) table (4-35)in the breast tumors patients the P-value of the genotypes frequencies of TLR4 3725 G / C gene were no significantly wherers patients with CC more affected by breast tumors comparison with patients having genotype GC(Odd ratio 3.20 , 2.24).

**Table (4-35) genotype frequency of.**

| Genotype TLR4 3725 G / C | Malignant tissue | Benign tissue | P value | OR CL 95% |
|---|---|---|---|---|
| **GG** | 16(67%) | 20(44%) | | |

| | | | | |
|---|---|---|---|---|
| **GC** | 5(2%) | 14(30%) | g 0.18 | 2.24(0.66-7.54) |
| **CC** | 3(2%) | 12(26%) | g 0.10 | 3.20( 0.76-13-31) |
| **Total** | 24 | 46 | | |
| **Alleles frequency** | | | | |
| **G** | 37(0.77) | 54(0.59) | | |
| **C** | 11(0.23) | 38(0.41) | 0.03* | 2.36( 1.07-5.21) |

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