Acknowledgment

Praise is due to Allah, Lord of the whole creation. Mercy and Peace are on the Prophet Mohammad and his relatives and companions .

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Sara Salih

3. Results and Discussion.

3.1. General Description of Clinical and pathologic characteristics of the Studied Samples

3.1.1 Differentiation of mean age between patients and control group

This study included ٧٠ Iraqi women with breast tumor; ٥٠ women with malignant breast tumor include ١٧patients under 40 years of age and ٣٣patients their age was over 40 years. Others 20 patients with benign tumor were included among them 12 were under age 40 years and 8 were over 40 years. The healthy group included 25women with 11 subjects less than 40 years old and 14 subject were over 40 years old. The mean age of malignant breast cancer patients which enrolled in this study was (52.13 ± 9.61) years while the mean of age of the benign tumor patients (38.58 ± 7.95) years. In control group the mean age was (41.28 ± 13.10) years.

Distributing the patients into age groups revealed that most of malignant breast tumor patients were clustered at the age group ≥ 40 years (٦٦%), while in benign breast tumor patients; such cluster was observed at the age group ≤ 40 years (60.0%). Chi-square analysis of these observations revealed a significant difference at $P \leq 0.01$. As shown in table (3-1).

Table (3-1) Distribution of malignant breast cancer, benign tumor patients and control subjects according to age.

Age group (year)	Patients	Benign	Apparently normal	Total
<40	١٧ (٣٤%)	12 (60%)	11 (44 %)	46 (38.3 %)
40>	٣٣ (٦٦ %)	8 (40 %)	14 (56 %)	74 (61.7 %)
Total	٥٠ (62.5 %)	20 (16.7 %)	25 (20.8 %)	120 (100 %)
Chi-square	٩.١٤٨ **	7.250 **	4.391 *	9.206 **
P-value	0.00٣٢٥	0.0142	0.0447	0.00782
* (P<0.05), ** (P<0.01).				

The risk of breast cancer incidence is increase in middle-aged women (after 40 years) and elderly than in young women (Edwards *et al*., 2002).

Human cancer is primarily an age-related disease that strikes when an individual is 50 years of age or older. The age of the individual and the time element are important largely because the formation of a tumor is a multistep process that takes many years to complete. Cancers are age-related because our cells change with time, becoming more susceptible to genetic damage and less capable of dealing with the damage when it does occur. This problem is believed to be due, in large measure, to a reduction in the ability of our immune system to track down and destroy abnormal cells as they appear; the body's diminished immune response gives those cells time to evolve into a potentially lethal cancer(Howlader *et al*., 2014).

The increased incidence of cancer in the age 50 and older is also coincidental with the onset of sexual senescence in women. It is quite possible that the hormonal changes that occur during this period contribute to increased susceptibility to breast cancer. Age related hormonal changes are primarily concerned with a shift in the ratio of estrogen to testosterone (E/T ratio) in women. Young women have a high estrogen/testosterone ratio (a lot of estrogen, very little testosterone). Estrogen levels drop dramatically in women after menopause. As a consequence, women approach a similar E/T ratio between the ages of 50 and 80, which are thought to influence the rate at which genetic instability occurs (Amita, 2009). In addition, many scientists believe the shift in the E/T ratio is largely responsible for the weakening of immune system, leading to the increased occurrence not only of cancer but of many other diseases as well (Joseph, 2014).

Carcinoma of breast occurs at any age after menarche but is not very common in women under 30 years of age; thereafter, the incidence increases steadily (Al-Sahab *et al*., 2010) The result of present study also agreed with Mohamed (2010) that revealed high frequency of breast cancer occur at age between 40-49 and the mean age of breast cancer patient 48.7 years and the high frequency of breast cancer incidence was at age 40-4 (Al-Anbari, 2009).

Incidence of breast cancer in female strongly related to age, however, highest incidence rates being in older women. the occurrence of the breast cancer in young women between 25 and 35 years approximately 2% and 11% between 35 and 45 years of age (Ries *et al*., 2008).

3.2. Molecular study

Nanodrop results indicated that the extracted genomic DNA was variable in concentration as it ranged from 20 to 300 ng/µl. Purity of DNA was also variable as some samples exceeded or fell below the standard purity limits (~2) depending on the freshness of blood and tissue samples. Agarose gel electrophoresis was used following Nanodrop to confirm the presence and integrity of extracted DNA.

3.2.1. Molecular detection of TLR4 mutation.

In the present study five sets of primer that designed using National Center of Biotechnology Information (NCBI Primer-Design online tool) were used to amplify four exons (2, 3, 4) in the TLR4 gene by using conventional Polymerase Chain Reaction (PCR) technique. The conditions of PCR were standardized.

The one set primers used in this PCR to amplify TLR4 gene (exon 2,3) product length which result from PCR amplification was appeared at 967 bp which show in figure(3-1).

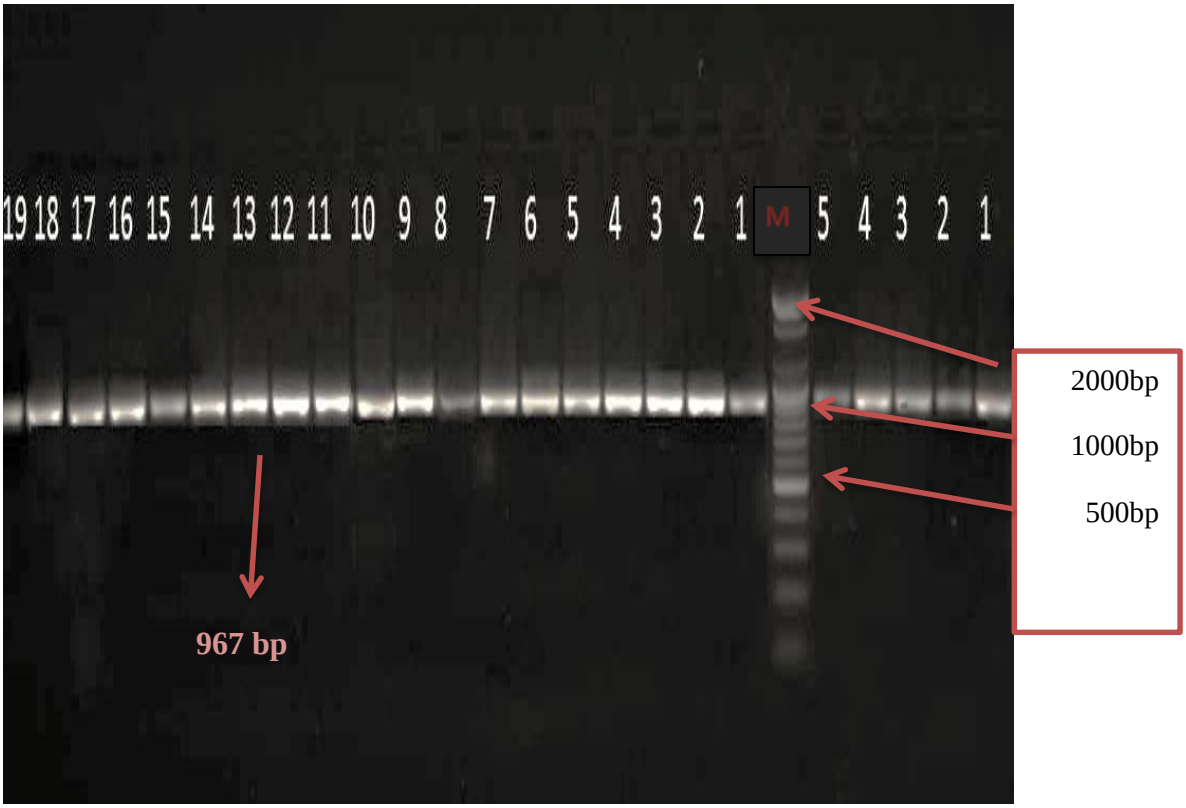

Figure (3-1): Gel electrophoresis for gene TLR4 (exon 2,3) PCR products visualized under U. V light after staining with ethidium bromide the agarose gel 1%, 10 minutes at 100 V and then lowered to 70 V, 50 minutes. M: 2000 bp marker; from right of the marker, lane 1-5: healthy individuals (controls); From left of the marker, 1-15: breast cancer patient lane; 16-19: from benign breast tumor. The size of product is 967 bp.

Second and third primer set that specific for TLR4 (exon4) the length of result product 919 and 720 bp respectively. As shown in figure from (3.3) .

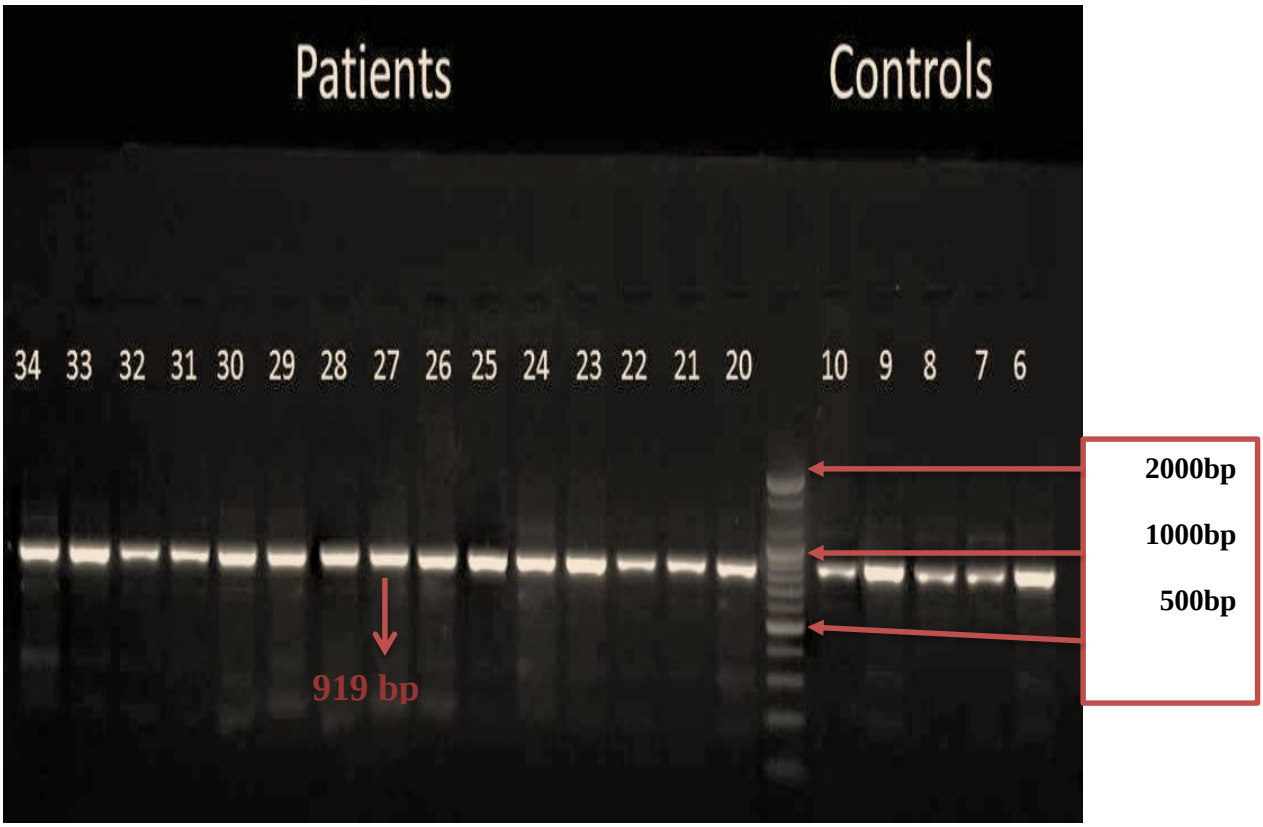

Figure (3-2): Gel electrophoresis of third primer for gene TLR4 (exon 4) PCR products visualized under U. V light after staining with ethidium bromide, the agarose gel 1%, 10 minutes at 100 V and then lowered to 70 V, 50 minutes. M: 2000 bp marker. From right of the marker 6-10 for control and from left of marker lane 20-25 benign breast tumor lane; 26-34: from breast cancer patients. The size of products 919 bp

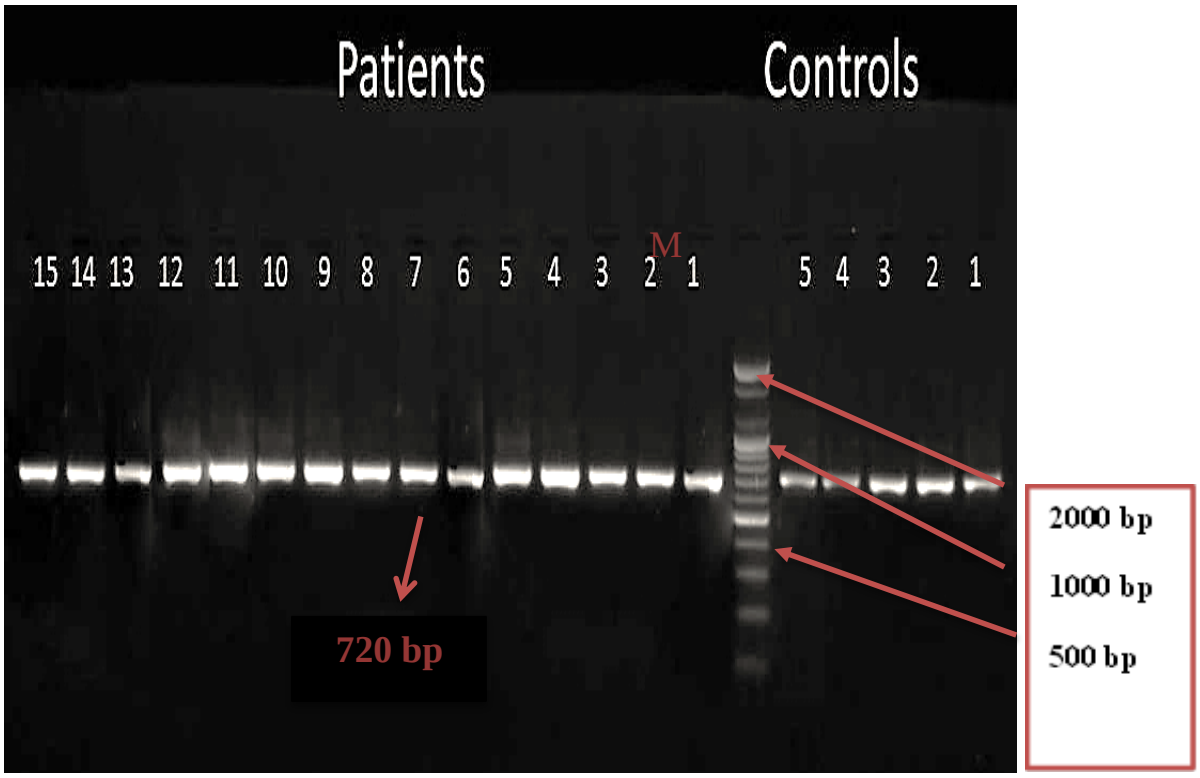

Figure (3-3): Gel electrophoresis of fourth primer for gene TLR4 (exon 4) PCR products visualized under U. V light after staining with ethidium bromide the agarose gel 1%, 10 minutes at 100 V and then lowered to 70 V, 50 minutes. M: 2000 bp marker; from right of the marker, lane 1-5: healthy individuals (controls); From left of the marker, 1-10: breast tumor lane; 11-19: from breast cancer patients. The size of proudcutes720 bp

3.2.2. Mutations detection by the DNA sequencing technique of TLR4

Automated DNA sequencing results in a computer output represented by a chromatogram (also called electropherogram) which is the visual representation of a DNA sequence containing nucleotide sequences and peaks. Good sequence generally begins roughly around base 20, and is represented by tall distinct peaks that have little overlap. Mutations in TLR4 gene were not detectable during

diagnosis by conventional PCR. For this reason the PCR products of TLR4 patients were screened by sequencing. PCR products were purified in preparation for sequencing. The sequences were analyzed to detect the presence of polymorphisms for TLR4 gene and compared with the international database on the National Center for Biotechnology Information (NCBI). Sequencing of TLR4 gene alignment using BLAST(Basic Local Alignment Search Tool) in which DNA sequence obtained in this study were compared with human reference TLR4 gene sequence (NG_018920.2). Alignment using BLAST tool made it easy to detect and locate mutations along this gene. Expect values (0.0) and BioEdit showed that the 99% similarities of malignant and healthy and benign samples.

The current study utilized a forward and reverse primer for sequencing TLR4 gene of sample of breast cancer patients. It was found that the mutations were found around all TLR4 gene regions involved in this study, i.e., (exon2, exon3 and exon4). According to NCBI, this streach contains 21 mutations. The mutations frequency was different among the four studied regions of the gene. Table (3-3) shows the details of the number of these mutations with their percentage. It is obvious that the highest mutations number was in exon 3, which was 11 mutations and this number is significant as the p-value of it is 0.0001, while the less mutation number found in exon 2 which is 6 and in exon4 which was 4 with p-value 0.0001 for each exon that is also significant. On the other hand, no mutations were detected in exon 1. It is clear that many samples showed mutations at different regions of the gene. Such sequencings of the coding regions of the amplified product of the TLR4 gene was done seeking for the detection of any mutations within these sequences related to breast cancer patients and the relation between the mutation was seen. The percentages of mutation types that displayed substitution 57.14% , deletion 33.33 % and insertion 9.52 % were present in

breast cancer patients, details in table (3-3) .The differences in this study were significant (P<0.01).

Table (3-2) Distribution of mutations of TLR4 in breast cancer patients.

Type of Polymorphism	Exon 2	Exon3	Exon 4
Deletion	5 83.34%	1 9.1%	1 25%
Insertion	0 00.00%	0 00.00%	2 50%
Substitution	1 16.66%	10 90.90%	1 25%
Total	6 (100%)	11 (100%)	4 (100%)
Chi square	15.00 **	14.07 **	15.00 **
P – value	0.0001	0.0001	0.0001

** (P<0.01), NS: Non-significant

Table (3-3): Percentage of mutation types in TLR4 gene in breast cancer patients.

Type of mutation	Percentage
Substitution	12(57.14%)
Deletion	7 (33.34%)
Insertion	2 (9.52%)

** ($P<0.01$).

The mutations observed within the exon 3 of TLR4 gene were compared to the healthy samples, all mutation in this exon were observed in malignant breast cancer patients while no mutation was detected in benign breast tumor patients and control,. A alignment with the TLR4 gene of Homo sapiens from the Gene Bank was done, 100% compatibility of that gene of healthy samples with standard genes of Gene Bank results are shown in figure .The mutation that we observed within exon 3 show that the samples of patients have 99% identities with the wild type . The exon 3 of the TLR4 gene of breast cancer patients have many mutation, 11 mutations which in exon 3 are A/G in position 673, A/G in position 609, also in position 578 A/G, the TTT/ GGC in position 84, 85, and 86 but in position 103 the T changed to G .In position 164 the A was deleted .In position 79-80 TC change to CA, The other SNP in this exon arein position 82 T/ G, as shown in figure (3-4, 3-5 and 3-6) and (3-7 a, b).

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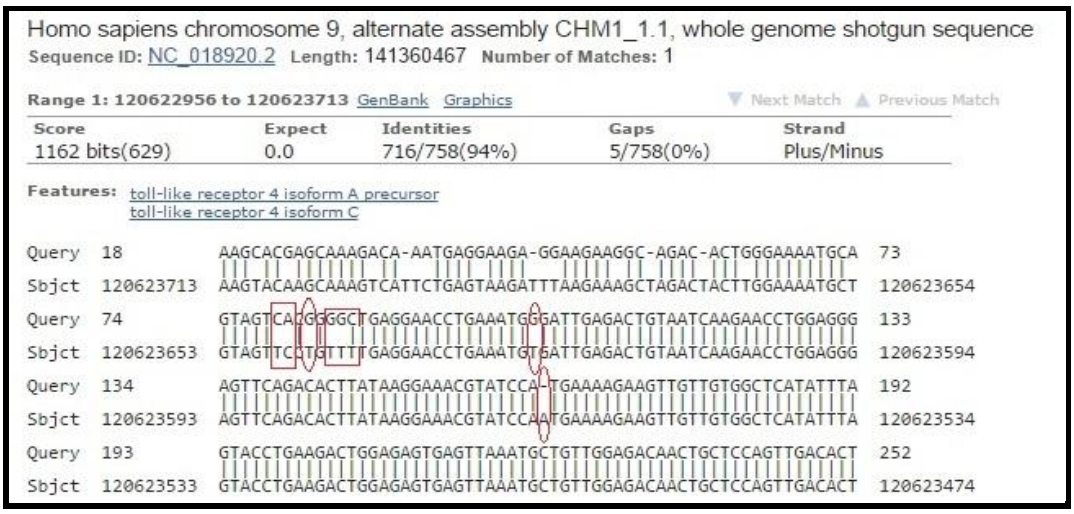


Figure (3-4) NCBI of breast cancer patient (exon 3) of TLR4

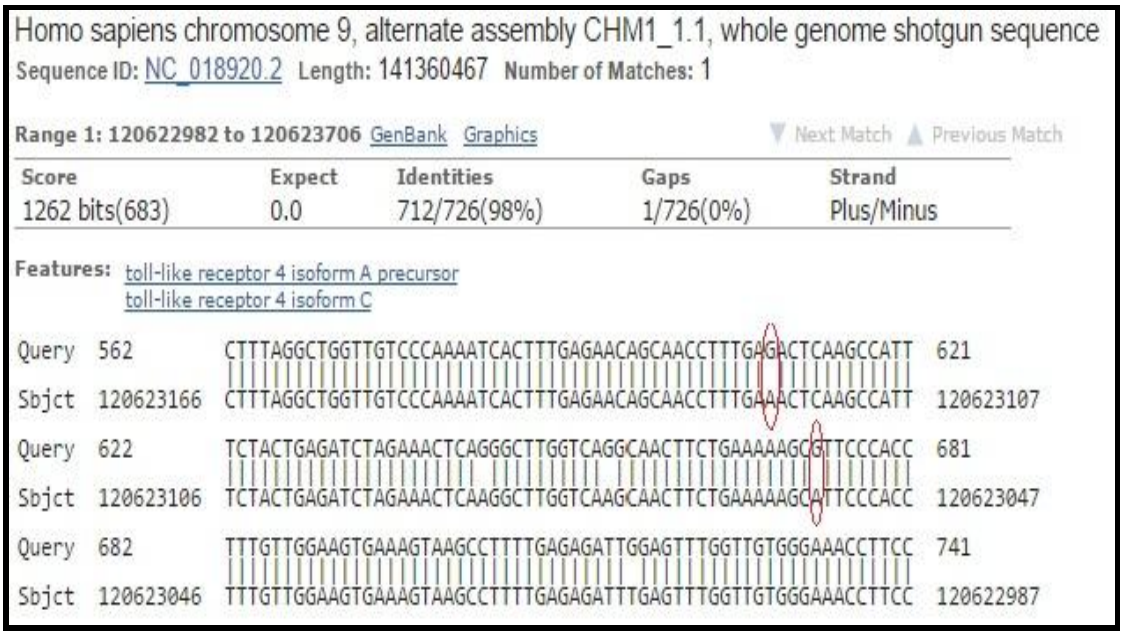


Figure (3-5) NCBI of breast cancer patient (exon 3) of TLR4

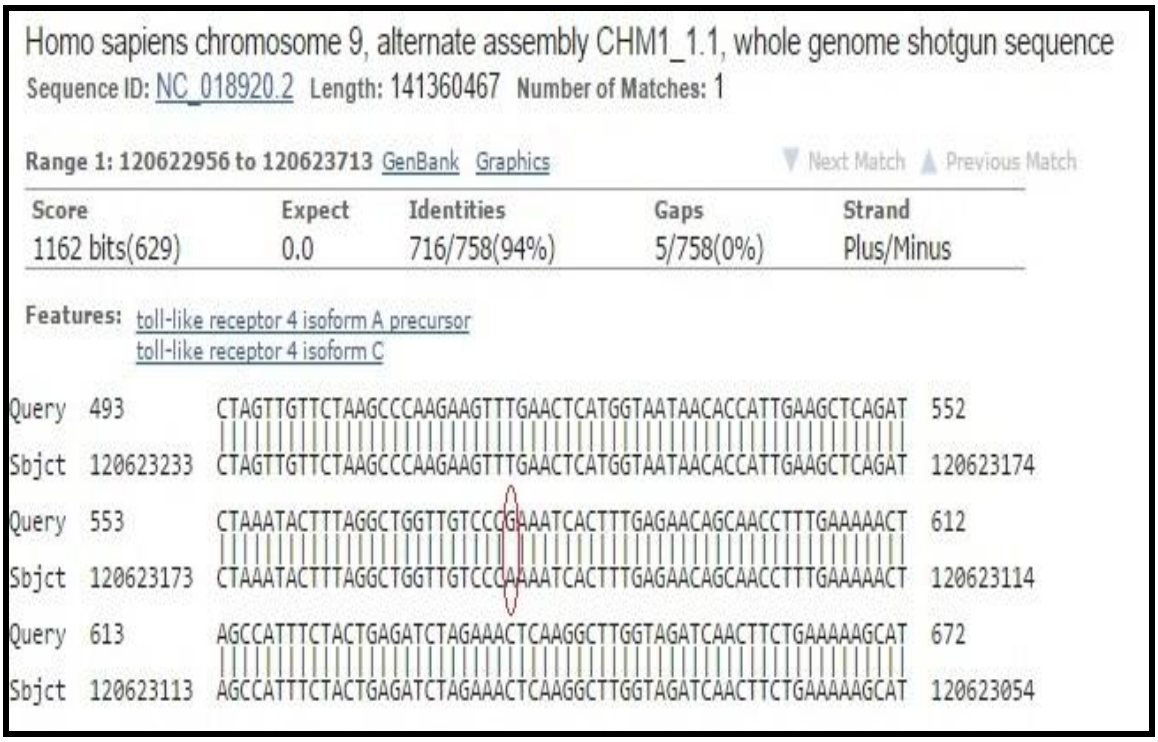
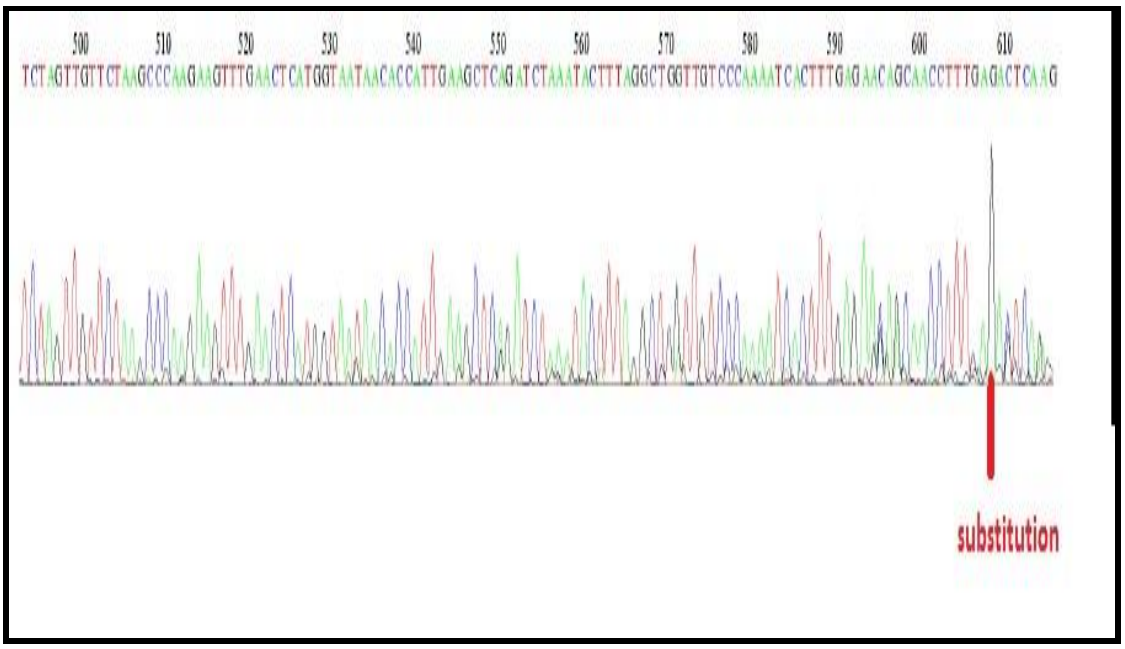


Figure (3-6) NCBI of breast cancer patient (exon 3) of TLR4

(A)



(B)

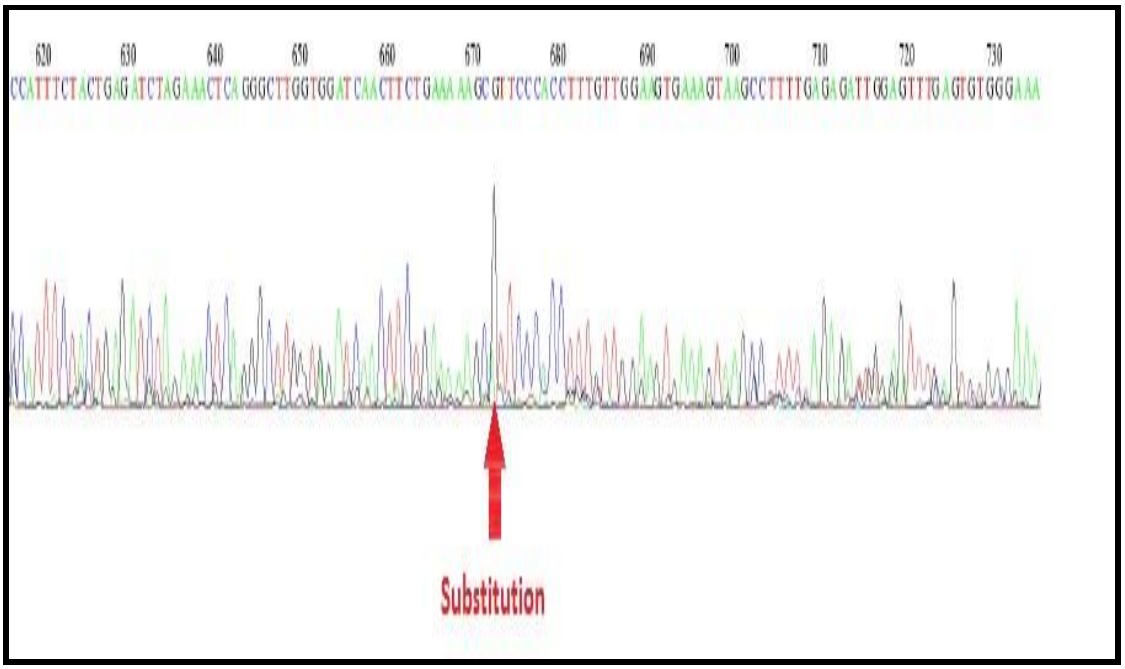
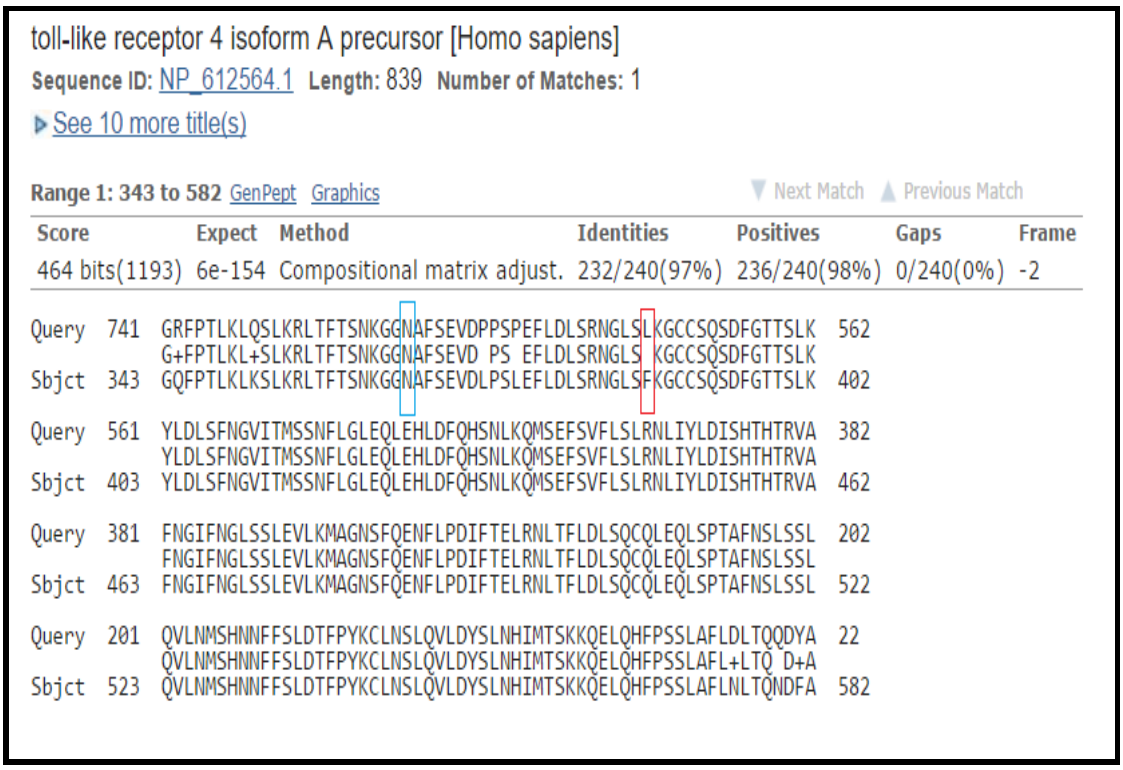


Figure (3-7 a, b) A and B: A chromatogram of breast cancer patients of TLR4 gene exon 3

The amino acid that resulted from the changes by the mutations are Phe change to Leu in position 609, in position 673 Asn not changed as show in figure (3-8) ,in 103 convert Cys to Trap, in 578 convert Gln to Arg ,while in position 79 Val not changed. In position 80 and 82 Prol change to Thr , Val convert to Gly in position 84 and 85 . Finally, In 164 Met converted to Stop codon .


toll-like receptor 4 isoform A precursor [Homo sapiens]

Sequence ID: NP_612564.1 Length: 839 Number of Matches: 1

See 10 more title(s)

Range 1: 343 to 582 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
464 bits(1193)	6e-154	Compositional matrix adjust.	232/240(97%)	236/240(98%)	0/240(0%)	-2

```
Query  741  GRFPTLKLQSLKRLTFTSNKGGNAFSEVDPPSPEFLDLSRNGLSLKGCCSQSDFGTTSLK  562
            G+FPTLKL+SLKRLTFTSNKGGNAFSEVD PS EFLDLSRNGLS KGCCSQSDFGTTSLK
Sbjct  343  GQFPTLKLKSLKRLTFTSNKGGNAFSEVDLPSLEFLDLSRNGLSFKGCCSQSDFGTTSLK  402

Query  561  YLDLSFNGVITMSSNFLGLEQLEHLDFQHSNLKQMSEFSVFLSLRNLIYLDISHTHTRVA  382
            YLDLSFNGVITMSSNFLGLEQLEHLDFQHSNLKQMSEFSVFLSLRNLIYLDISHTHTRVA
Sbjct  403  YLDLSFNGVITMSSNFLGLEQLEHLDFQHSNLKQMSEFSVFLSLRNLIYLDISHTHTRVA  462

Query  381  FNGIFNGLSSLEVLKMAGNSFQENFLPDIFTELRNLTFLDLSQCQLEQLSPTAFNSLSSL  202
            FNGIFNGLSSLEVLKMAGNSFQENFLPDIFTELRNLTFLDLSQCQLEQLSPTAFNSLSSL
Sbjct  463  FNGIFNGLSSLEVLKMAGNSFQENFLPDIFTELRNLTFLDLSQCQLEQLSPTAFNSLSSL  522

Query  201  QVLNMSHNNFFSLDTFPYKCLNSLQVLDYSLNHIMTSKKQELQHFPSSLAFLDLTQQDYA  22
            QVLNMSHNNFFSLDTFPYKCLNSLQVLDYSLNHIMTSKKQELQHFPSSLAFL+LTQ D+A
Sbjct  523  QVLNMSHNNFFSLDTFPYKCLNSLQVLDYSLNHIMTSKKQELQHFPSSLAFLNLTQNDFA  582
```

Figure (3-8) polypeptide product of exon 3.

Besides, there are other mutations that are detected in TLR4 gene in exon2. The mutations observed within the exon 2 are G/ A in position 849, deletion C in position 23. In position 30 deletions G, deletion A in position 865. Last mutations in exon 2 is deletion A in position 897and 820. These SNPs showed in figure (3-9and 10).

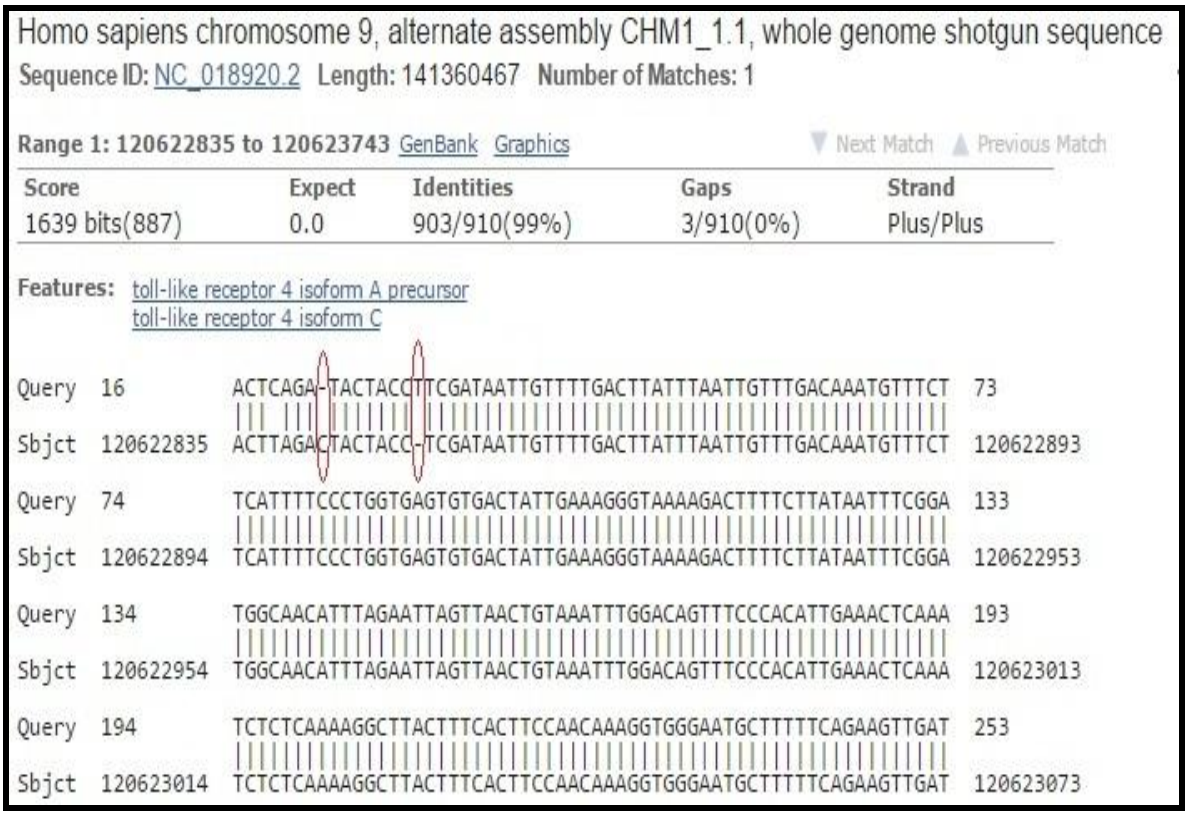


Figure (3-9) NCBI of breast cancer patient (exon 2) of TLR4

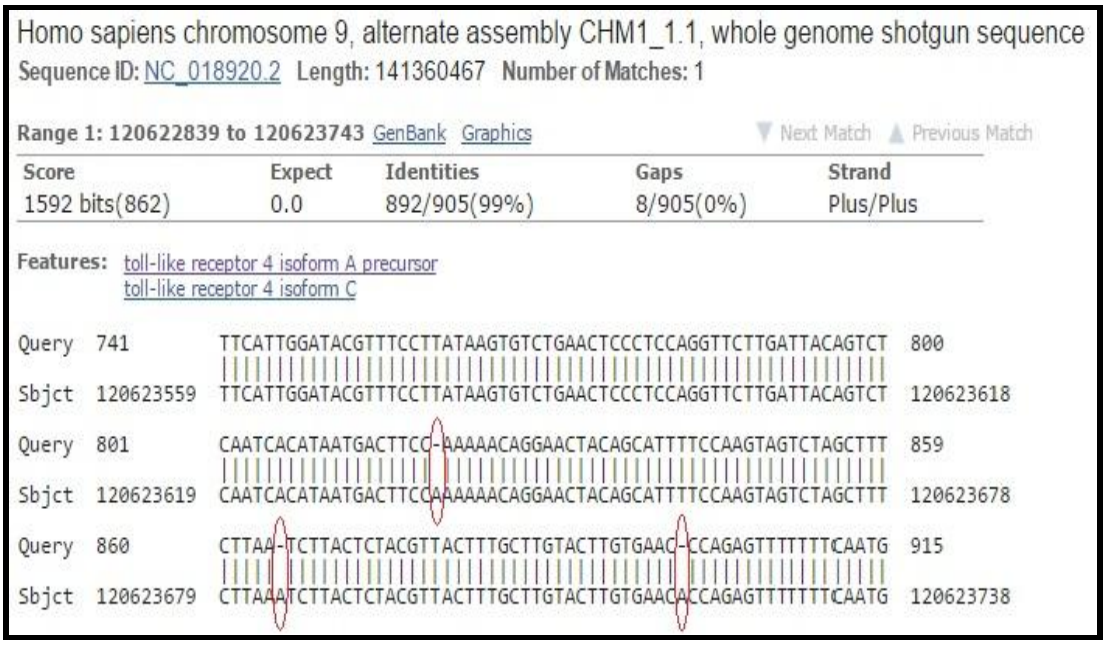


Figure (3-10) NCBI of breast cancer patient (exon 2) of TLR4

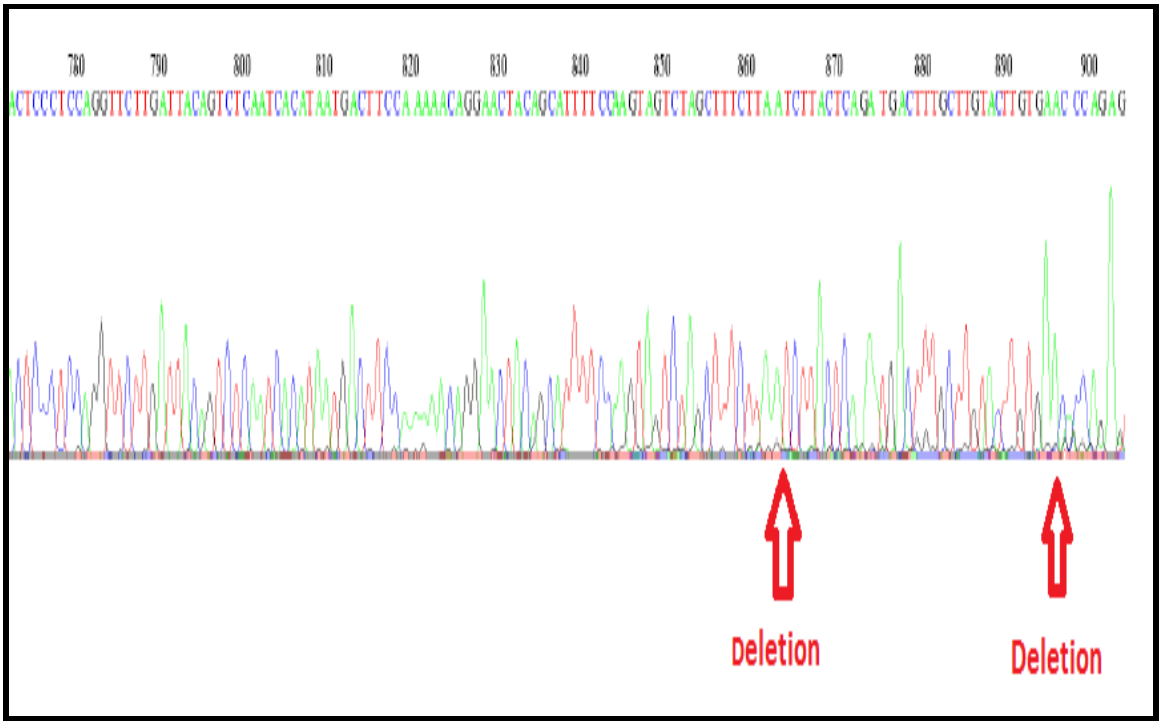
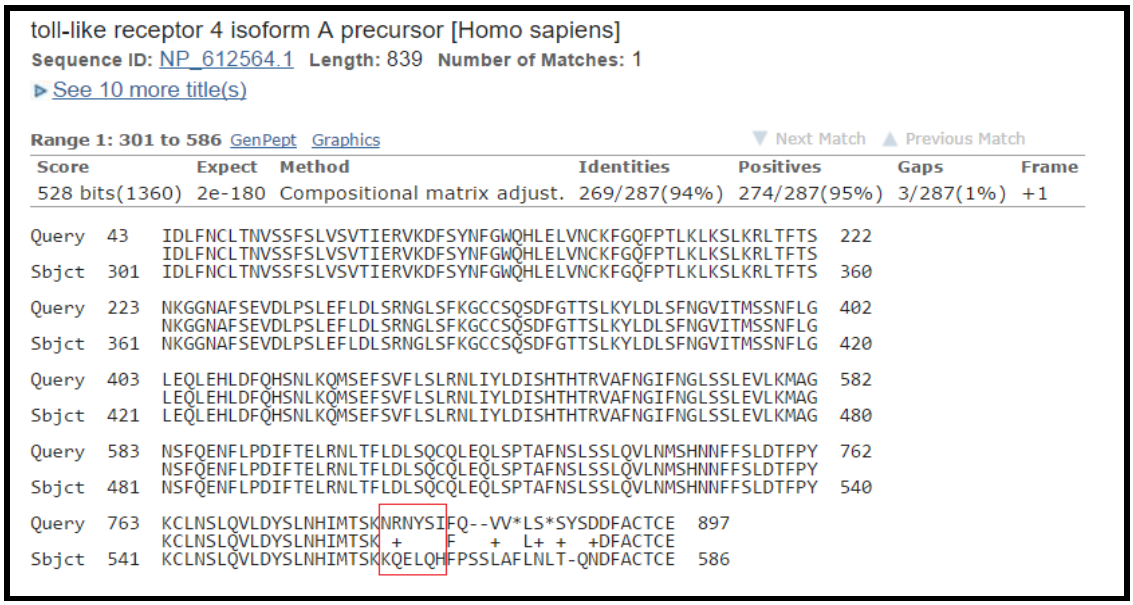


Figure (3-11) A chromatogram of breast cancer patients of TLR4 gene exon 2.

The amino acid that resulted from mutations within the exon 2 is Ser convert to Asn in position 849, in position 23and 30, Asn change to Ile in position 865. Last mutations in exon 2 is deletion A in position 897 His convert to Pro. While in position Lys do not changed as show in figure (3-12 and 13).



toll-like receptor 4 isoform A precursor [Homo sapiens]

Sequence ID: NP_612564.1 **Length:** 839 **Number of Matches:** 1

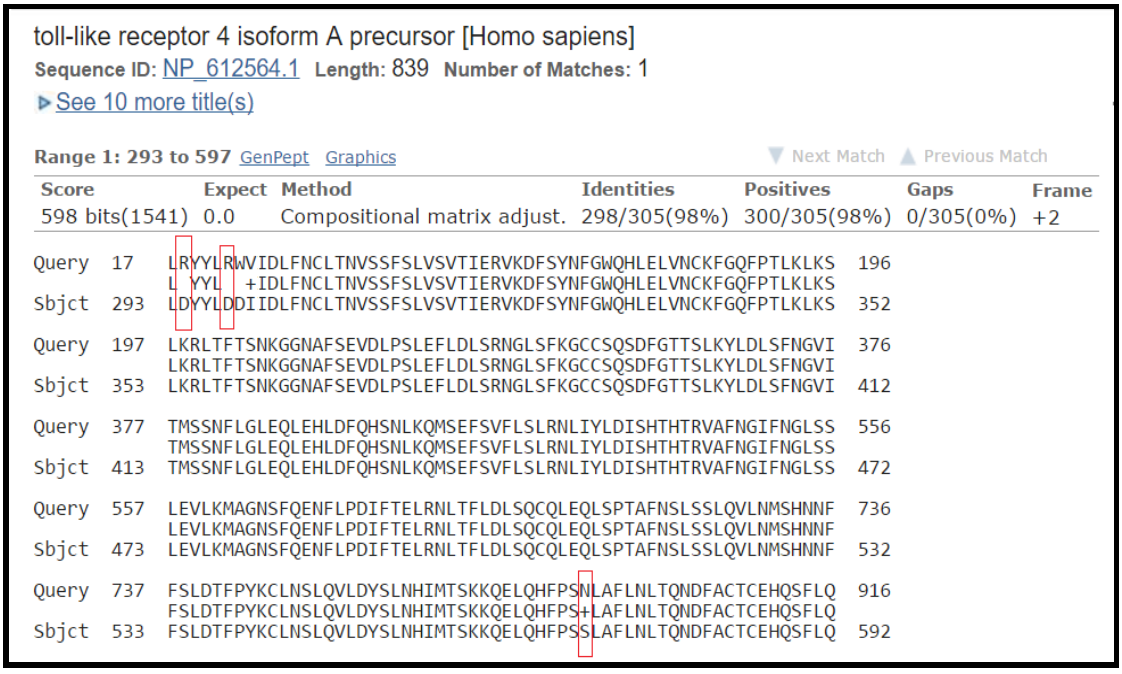
See 10 more title(s)

Range 1: 301 to 586 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
528 bits(1360)	2e-180	Compositional matrix adjust.	269/287(94%)	274/287(95%)	3/287(1%)	+1

```
Query  43   IDLFNCLTNVSSFSLVSVTIERVKDFSYNFGWQHLELVNCKFGQFPTLKLKSLKRLTFTS  222
            IDLFNCLTNVSSFSLVSVTIERVKDFSYNFGWQHLELVNCKFGQFPTLKLKSLKRLTFTS
Sbjct  301  IDLFNCLTNVSSFSLVSVTIERVKDFSYNFGWQHLELVNCKFGQFPTLKLKSLKRLTFTS  360

Query  223  NKGGNAFSEVDLPSLEFLDLSRNGLSFKGCCSQSDFGTTSLKYLDLSFNGVITMSSNFLG  402
            NKGGNAFSEVDLPSLEFLDLSRNGLSFKGCCSQSDFGTTSLKYLDLSFNGVITMSSNFLG
Sbjct  361  NKGGNAFSEVDLPSLEFLDLSRNGLSFKGCCSQSDFGTTSLKYLDLSFNGVITMSSNFLG  420

Query  403  LEQLEHLDFQHSNLKQMSEFSVFLSLRNLIYLDISHTHTRVAFNGIFNGLSSLEVLKMAG  582
            LEQLEHLDFQHSNLKQMSEFSVFLSLRNLIYLDISHTHTRVAFNGIFNGLSSLEVLKMAG
Sbjct  421  LEQLEHLDFQHSNLKQMSEFSVFLSLRNLIYLDISHTHTRVAFNGIFNGLSSLEVLKMAG  480

Query  583  NSFQENFLPDIFTELRNLTFLDLSQCQLEQLSPTAFNSLSSLQVLNMSHNNFFSLDTFPY  762
            NSFQENFLPDIFTELRNLTFLDLSQCQLEQLSPTAFNSLSSLQVLNMSHNNFFSLDTFPY
Sbjct  481  NSFQENFLPDIFTELRNLTFLDLSQCQLEQLSPTAFNSLSSLQVLNMSHNNFFSLDTFPY  540

Query  763  KCLNSLQVLDYSLNHIMTSKNRNYSIFQ--VV*LS*SYSDDFACTCE  897
            KCLNSLQVLDYSLNHIMTSK +    F   +  L+ +  +DFACTCE
Sbjct  541  KCLNSLQVLDYSLNHIMTSKKQELQHFPSSLAFLNLT-QNDFACTCE  586
```

Figure (3-12) polypeptide product of exon 2.



toll-like receptor 4 isoform A precursor [Homo sapiens]

Sequence ID: NP_612564.1 **Length:** 839 **Number of Matches:** 1

See 10 more title(s)

Range 1: 293 to 597 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
598 bits(1541)	0.0	Compositional matrix adjust.	298/305(98%)	300/305(98%)	0/305(0%)	+2

```
Query  17   LRYYLRWVIDLFNCLTNVSSFSLVSVTIERVKDFSYNFGWQHLELVNCKFGQFPTLKLKS  196
            L YYL  +IDLFNCLTNVSSFSLVSVTIERVKDFSYNFGWQHLELVNCKFGQFPTLKLKS
Sbjct  293  LDYYLDDIIDLFNCLTNVSSFSLVSVTIERVKDFSYNFGWQHLELVNCKFGQFPTLKLKS  352

Query  197  LKRLTFTSNKGGNAFSEVDLPSLEFLDLSRNGLSFKGCCSQSDFGTTSLKYLDLSFNGVI  376
            LKRLTFTSNKGGNAFSEVDLPSLEFLDLSRNGLSFKGCCSQSDFGTTSLKYLDLSFNGVI
Sbjct  353  LKRLTFTSNKGGNAFSEVDLPSLEFLDLSRNGLSFKGCCSQSDFGTTSLKYLDLSFNGVI  412

Query  377  TMSSNFLGLEQLEHLDFQHSNLKQMSEFSVFLSLRNLIYLDISHTHTRVAFNGIFNGLSS  556
            TMSSNFLGLEQLEHLDFQHSNLKQMSEFSVFLSLRNLIYLDISHTHTRVAFNGIFNGLSS
Sbjct  413  TMSSNFLGLEQLEHLDFQHSNLKQMSEFSVFLSLRNLIYLDISHTHTRVAFNGIFNGLSS  472

Query  557  LEVLKMAGNSFQENFLPDIFTELRNLTFLDLSQCQLEQLSPTAFNSLSSLQVLNMSHNNF  736
            LEVLKMAGNSFQENFLPDIFTELRNLTFLDLSQCQLEQLSPTAFNSLSSLQVLNMSHNNF
Sbjct  473  LEVLKMAGNSFQENFLPDIFTELRNLTFLDLSQCQLEQLSPTAFNSLSSLQVLNMSHNNF  532

Query  737  FSLDTFPYKCLNSLQVLDYSLNHIMTSKKQELQHFPSNLAFLNLTQNDFACTCEHQSFLQ  916
            FSLDTFPYKCLNSLQVLDYSLNHIMTSKKQELQHFPS+LAFLNLTQNDFACTCEHQSFLQ
Sbjct  533  FSLDTFPYKCLNSLQVLDYSLNHIMTSKKQELQHFPSSLAFLNLTQNDFACTCEHQSFLQ  592
```

Figure (3-13) polypeptide product of exon 2.

The four mutations exon 4 of the gene are deletion A in position 144 convert Ser to Val , and insertion A converts Ser to Tyr in position 83. In position 33 insertions G that convert Phe to Leu. Finally, in position 28 A changed to T which convert Glu to Val.

Breast cancer is one of the leading causes of cancer death in women (Hammoudeh *et al*., 2016). Studies have shown that genetic variants may be involved in the process of breast carcinogenesis (Yan *et al*., 2016). Several mutations within the TLR genes have been associated with tumorigenesis (Zidi *et al*., 2013). Recent evidence had suggested the link between mutated TLR4 and breast cancer (Muscatel *et a*l., 2016).

Theodoropoulo *et al*., (2012) reported that TLR4 gene mutation might increase susceptibility to breast cancer development. TLR4 are mainly expressed in macrophages, dendritic cells and other innate immune cells. Intriguingly others have found that TLR4 was highly expressed in breast cancer cells (Bhatelia *et al*., 2014).

However, Pullin *et al*., (2010) cleared that TLR4 mutations have been shown to be important in many types of cancer with major inflammatory components, including breast and ovarian cancer.

Haricharan *et al*., (2015) revealed that association of breast carcinoma with TLR4 mutations, which is the most commonly mutated (> 50%) gene related with some cancer types.

Malignancies with frequent mutation of TLR4 such as ovarian serous carcinoma, breast and bladder cancers all appear to retain high TLR4 levels. As cancers with TLR4 mutations are much more aggressive and difficult to treat,

TLR4 represents a broadly valuable target for these patients (Mehmeti *et al.*, 2015).

Liao *et al.*, (2012) suggested that TLR4 mutations play important roles in the migration of cancer cells TLR4 prompts human breast cancer cells invasiveness and leads to induction of pro-inflammatory and chemokine genes. Clinical studies showed a significant association of high TLR4 expression with lymph node metastasis and local cancer proliferation (Yang *et al*., 2014)

TLR signaling had been shown to regulate cell death and increase expression of the anti-apoptotic proteins. Mutated TLR4 signaling can help tumor escape immune surveillance and also enhance tumor cell metastasis (Ling *et al.*, 2014). The specific role of mutated TLR4 mediated cellular process like growth, migration and resistance/sensitivity to death lead to metastasis of the breast cancer. Increasing evidence suggests that the neoplastic process may impede TLR signaling pathways to favor cancer progression (Matijevic and Pavelic, 2010).

Collectively, mutations in TLR4 gene appears to be associated with increased susceptibility to breast cancer could act as a prognostic marker for the breast cancer (Sandholm and Selander, 2014).

Immune system plays a crucial role not only in defense against microbial infections but also in control and surveillance of malignant cells. Immune cells can scan tissues with the object to remove newly formed malignant cells before they turn into fully formed tumors (Igney and Krammer, 2002).

TLRs are expressed on cells of the immune system but there is growing evidence that TLRs are also expressed on tumor cells, where they may influence tumor growth and host immune responses (Iwasaki *et al* ., 2004). Toll-like receptor

(TLR) 4-mediated signaling has been implicated in tumor cell invasion, survival, and metastasis in a variety of cancers, however, breast cancer is one of these cancers (Huan, *et al*., 2014).

*3.2.3. Molecular detection of MYD88 by PCR technique

In this study specific primer was designed using the NCBI Primer-Design online. In order to amplify MYD88 gene (exon 1, 5) by using a routine PCR technique specific for exon 1 MYD88 gene from NCBI primer design with product length 606bp. All samples appeared at the band in PCR technique. 15(20 %) malignant breast cancer patients show no band, as show in figure (3-14).

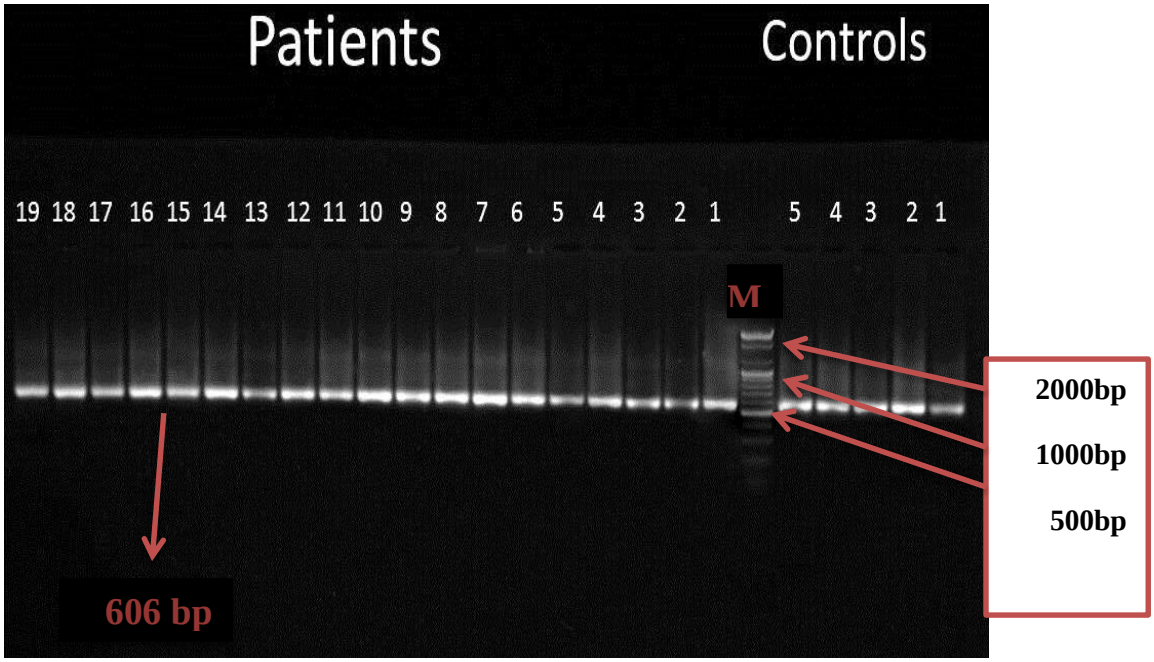


Figure (3-14): Gel electrophoresis for gene MYD88 gene (exon 1) PCR products visualized under U. V light after staining with ethidium bromide the agarose gel 1%, 10 minutes at 100 V and then lowered to 70 V, 50 minutes. M: 2000 bp marker; ; from right of the marker, lane 1-5: healthy individuals (controls); From left of the marker, 1-10: benign breast tumor lane; 11-19: from breast cancer patients. The size of products 606 bp.

The second set primers used in this PCR to amplify MYD gene (exon 1,5) product length which result from PCR amplification was appeared at (877) pb the results show10(5.33 %) malignant breast cancer patients and 2 (2.66%) benign breast tumor show no band , as show in fig (3-15).

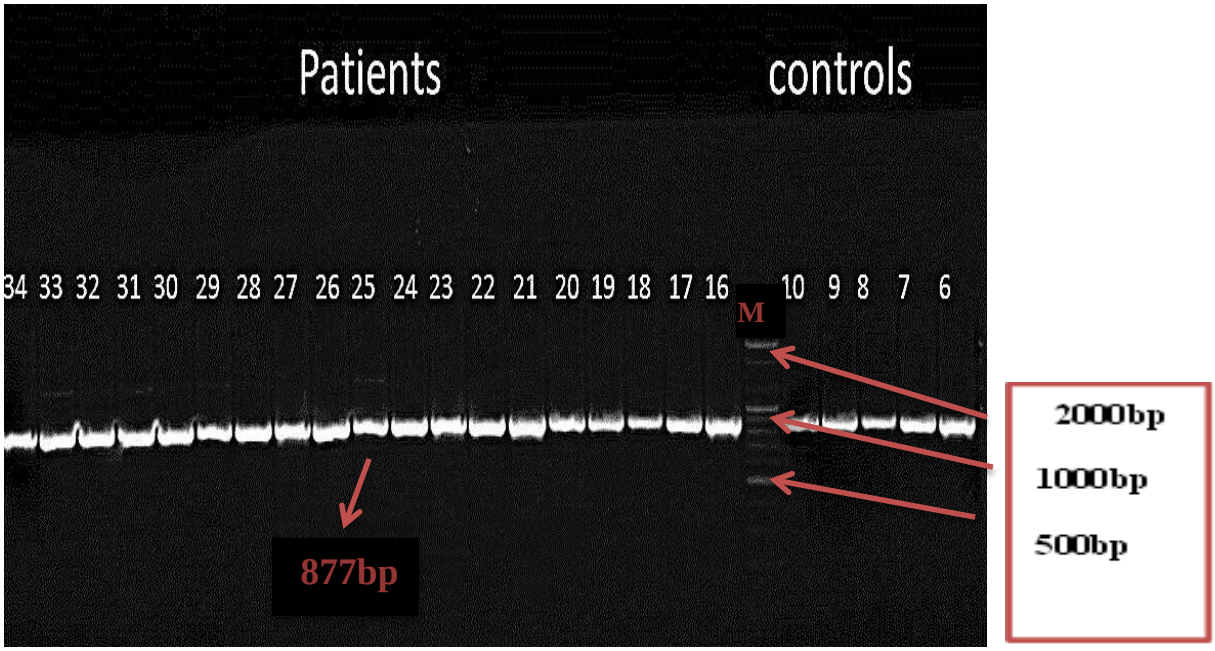


Figure (3.15): Gel electrophoresis The fourth primer for gene MYD88 (exon 5) PCR products visualized under U. V light after staining with ethidium bromide, the agarose gel 2%, 10 minutes at 100 V and then lowered to 70 V, 50 minutes. M: 2000 bp marker, from the left of the marker lane 20-34 blood samples of breast cancer patients. From the right of marker 6-10 for control. The size of product is 877 bp.

3.2.4. Mutations detection by the DNA sequencing technique of MYD88

The sequences were analyzed to detect the presence mutations for MYD88 gene and compared with the international database on the National Center for Biotechnology Information (NCBI). Sequencing of MYD88 gene alignment using BLAST(Basic Local Alignment Search Tool) in which DNA sequence obtained in this study were compared with human reference MYD88 gene sequence (NG_ Ref.018914.2). Alignment using BLAST tool made it easy to detect and locate mutations along this gene.

In exon 1 at position 512 G/ A, G/T at position 569. Finally, at position 104 A changes to G. Also in other region in same gene, exon1 have mutations as is clear in the Figure (3-16 and 17).

Figure (3-15) NCBI of breast cancer patient (exon 1) of Myd88 gene.

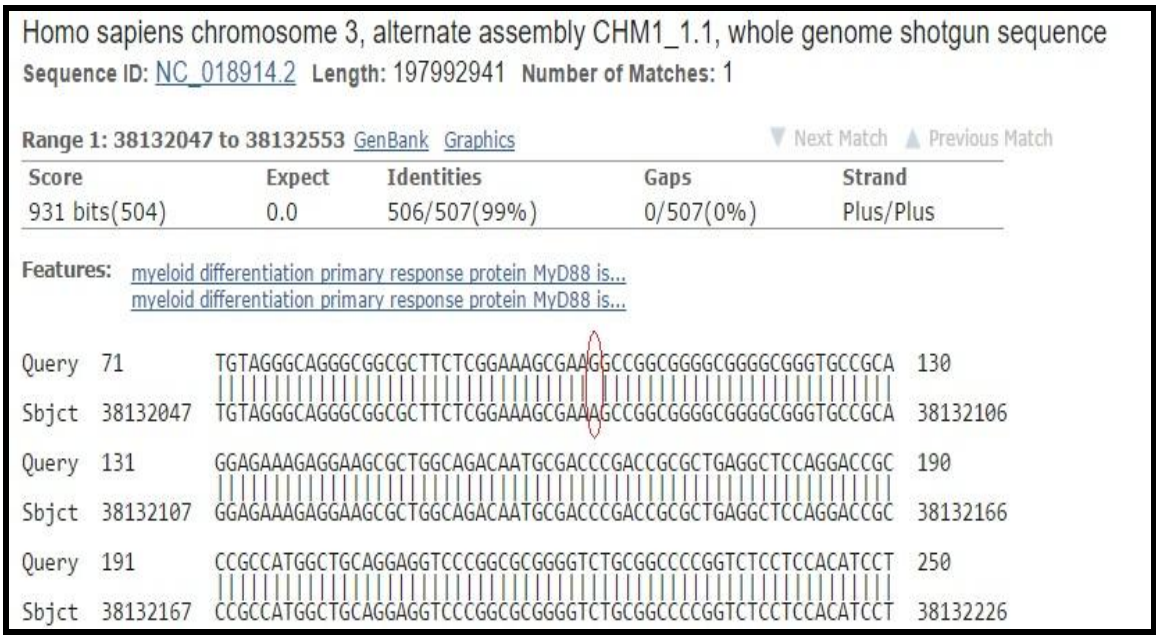


Figure (3-16) NCBI of breast cancer patient (exon 1) of Myd88 gene.

The amino acid that resulted from mutations within exon 1 His convert to Gln in position 512, in position 569 Arg / Leu, Leu change to Pro in position 104 as shown in figures (3- 17, 18).

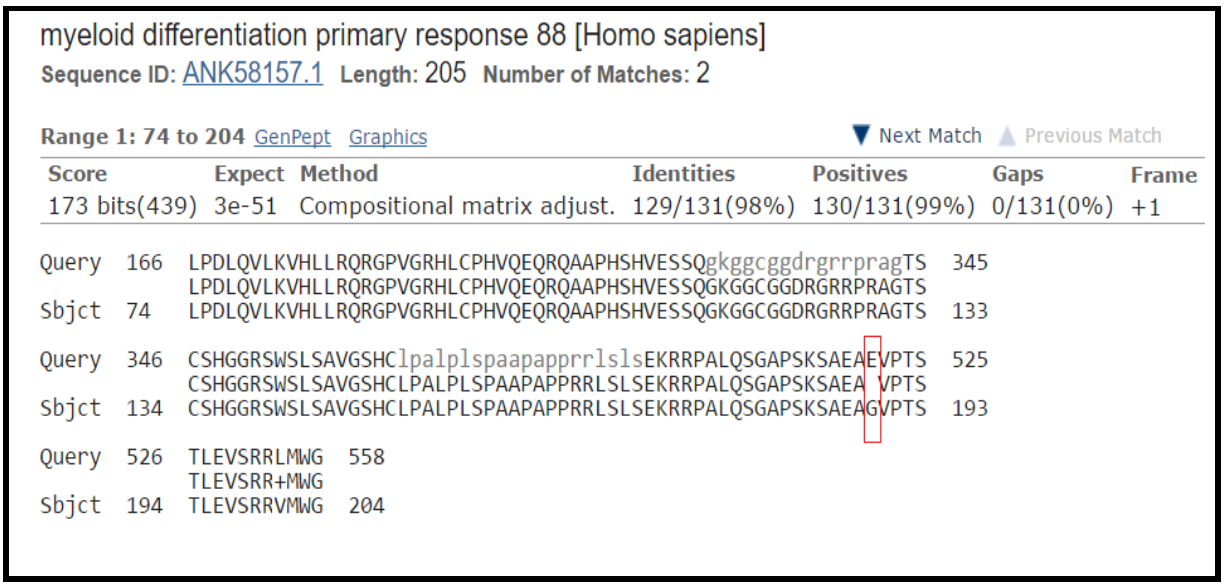


Figure (3-17) polypeptide product of exon 1of Myd88

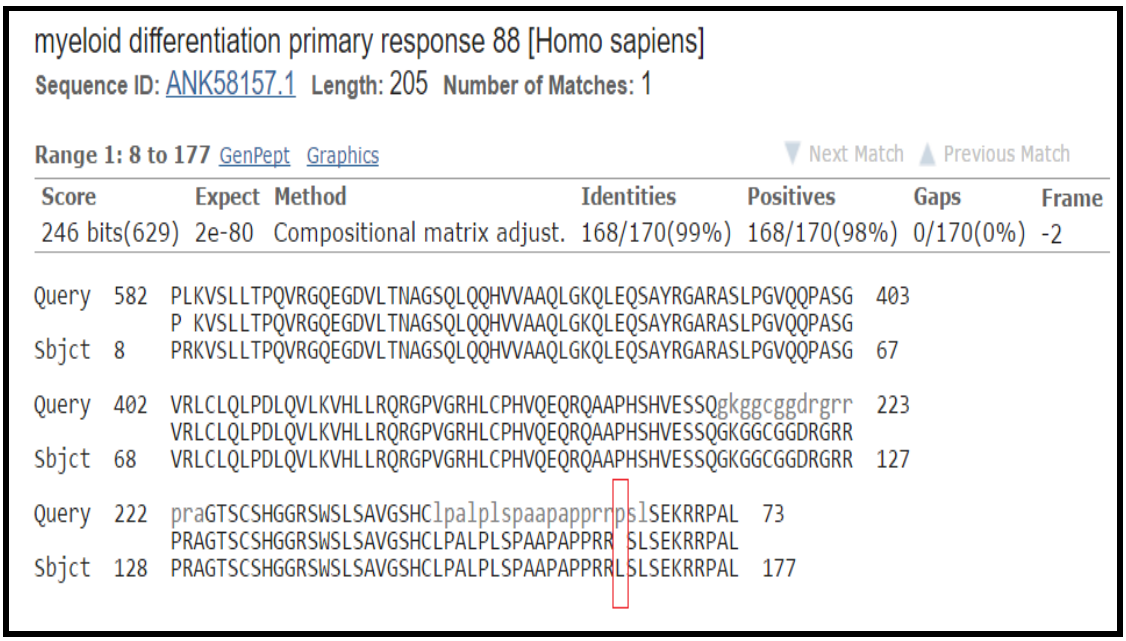


Figure (3-18) polypeptide product of exon 1of Myd88

The percentages of mutations types that displayed 100% substitution was present in breast cancer patients, details in table (3-5) .The differences in this study were significant (P<0.01)

Table (3-4): Percentage of mutations types in MYD88 gene in breast cancer patients

Type of mutation	Exon 1	Exon 5
Deletion	0 (0.00%)	0 (0.00%)
Insertion	0 (0.00%)	0 (0.00%)
Substitution	3 (100%)	0 (0.00%)
Total	3 (100%)	0 (0%)
Chi square	15.00 **	0.00 NS
P – value	0.0001	1.00

** (P<0.01), NS: Non-significant.

Table (3-5): Percentage of polymorphism types in MYD88 gene in breast cancer patients

Type of mutation	Percentage
Substitution	3(100%)
Deletion	0
Insertion	0
P – value	0.0113 **

** (P<0.01).

This gene encodes a cytosolic adapter protein that plays a central role in the innate and adaptive immune response. This protein functions as an essential signal transducer in the interleukin-1 and Toll-like receptor signaling pathways. These pathways regulate that activation of numerous proinflammatory genes (Loiarro *et al.*, 2013).

Braeuer *et al*., (2006) showed that MYD88 mutations were not identified in normal breast tissue samples, indicating that MYD88 may have diagnostic utility for breast carcinoma. Recent studies revealed MYD88 mutation in hematological malignancies and MYD88 overexpression in some solid cancers. MYD88 expression in normal cells differs depending on the organs. The data suggest that a gain of MYD88 expression in gastric cancers might play a role in cancer pathogenesis by activating oncogenic functions of MYD88.

In cancer cells, multiple genetic alterations (IRAK1 amplifications, MYD88 mutations, TLR4 mutations) can occur in the TLR4/MyD88 pathway resulting in an activation of this pathway (Ngo *et al*., 2013)

Martínez *et al*., (2014) revealed that the Variation in myeloid differentiation primary response gene 88 (MYD88) found in 90% of the breast cancer patients.

MYD88 mutations result in the chronic activation of TLR signaling pathways, thus the constitutive activation of the transcription factor NFκB to promote survival and proliferation of cancerous breast cells (Vacchelli *et al*., 2012).

Ning *et al*., (2011) suggested that Inappropriate activation of TLRs due to the somatic acquisition of gain-of-function mutations in the TLR adaptor protein MYD88has been implicated in many malignancies such as breast carcinoma.

MYD88 mutations are critical for high NFκB transcription factor activity .as well as, the hyper-phosphorylated isoform of IRAK1 was strongly associated with mutant form of MYD88, suggesting that this mutation is again-of-function mutation that leads to the constitutive activation of downstream IRAKs (Hanahan *et al*., 2011).

Lam *et al*., (2008) cleared that the effect of MYD88 mutation on breast tumor growth is confounded by the accumulation of other potential damaging mutations in the same malignant clones.

TLR-4 plays a significant role in gut innate immunity, protection and is involved in human breast cancer (Lubbad *et al*., 2009). Cantó *et al* 2006 reported that a marked increase of TNF-α response to TLR2 ligands correlated with a higher TLR4 expression in malignant breast carcinoma patients, indicating that an abnormal mechanism may provide an excess of inflammatory mediators during tumorgensis . This TLR signaling acts through a downstream regulator, MyD88, which initiates a signal transduction cascade leading to the induction of NF-κB (Cantó *et al*., 2006). Although TLRs signal transmit through the MyD88-dependent or -independent pathway, previous studies have indicated a significant role of the TLR-MyD88 pathway in cancer (Siddique and Khan, 2011). Aoyagi *et al* have

reported that mRNA levels of MyD88, TLR-4 and NF-κB are significantly increased in breast cancer patients (Aoyagi *et al.*, 2010). Therefore, MyD88 is considered one of the key molecules in the tumorgenesis of breast and an overexpression of MyD88 may promote the development of breast cancer.

3.4. Grading of breast cancer (World health organization grading system).

Pathologists describe cells as well differentiated (grade I), moderately differentiated (grade II), and poorly differentiated (grade III) as the cells progressively lose the features seen in normal breast cells (Benson *et al.*, 2003). Thirty samples of malignant breast tumor patients which enrolled in present study distributed according to grade of breast tumor .It was observed that more than 43.33% of malignant BT patient were classified as grade II (moderate differentiation), followed by grades III and I (40% and 16.33%, respectively). Such findings suggest that 43.33% of investigated patients were at an intermediate risk to develop metastasis as compared with low risk metastasis (low grade) and high risk metastasis (high grade) (Weigelt *et al*., 2005). There was significant ($P < 0.01$) difference in grade of tumor between malignant breast tumor patients and women with benign breast tumor. As show in table (3-6).

Table (3-6): Distribution of sample study according to grade of H& E for breast cancer patient.

Grade	Malignant		Chi-square value (χ^2)
	No.	%	
Grade 1 Well differentiation	5	16.67	**6.038 ****
Grade 2 Moderate differentiation	13	43.33	**6.544 ****
Grade 3 Poor differentiation	12	40.00	**10.783 ****
Total	30	100%	**---**
Chi-square value (χ^2)	**9.215 ****		**---**
**** (P<0.01).**			

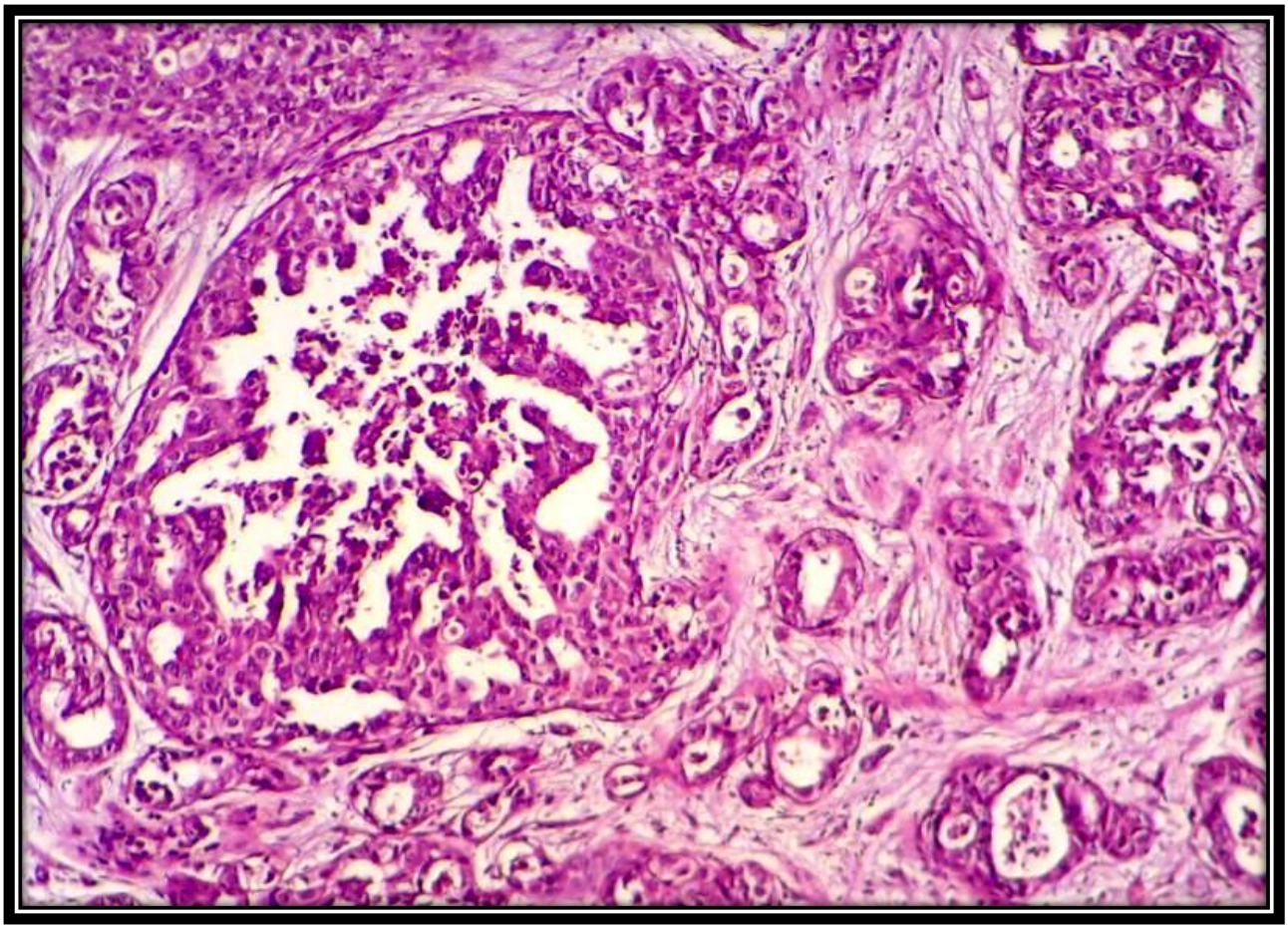


Figure (3-19): Sections of infiltrative ductal carcinoma of breast, Moderate differentiation, histological grade II. (X 40)

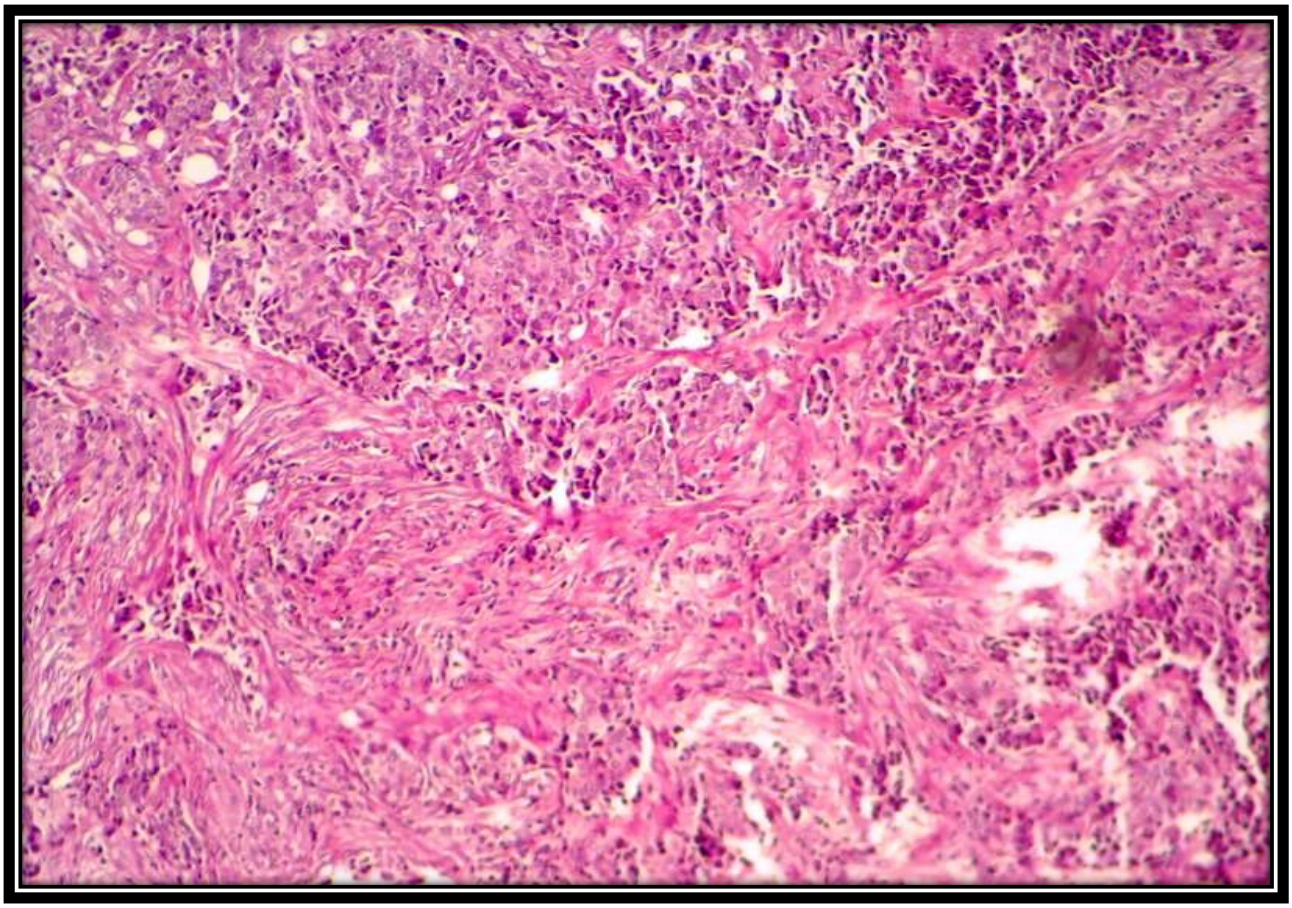


Figure (3-20): Sections of infiltrative ductal carcinoma of breast, Poor differentiation, histological grade III. (X 20)

Kim *et al*., (2007) were agreeing with this study in ovarian epithelial cancers of TLR-4. Another finding of the current study was the lack of significant correlation between TLR- 4 overexpression with grade and invasion, there was a complete agreement with D'Adhemar *et al*.,2014 in no correlation between grade and TLR-4 overexpression in breast cancer, and with Kim *et al*.,2012 in lack of correlation between grade and stage of breast carcinoma with TLR-4 but different result were obtained by Stopiglia *et al*.,2015 who demonstrated that TLR-4 immune reactivities were significantly lower in low-grade breast carcinoma than normal, and this may contribute to the high tumor relapse and progression rates. Moreover, Yuan *et al.,* 2013 which detected strong positive IHC staining of TLR-4for advanced-stage breast carcinoma whereas moderate or weak staining for early-stage tumors while, normal breast epithelia were generally negative for TLR4.

3.6. Immunohistochemical expression of Toll like receptor 4

Immunostaining was used to determine the positive expression of TLR4. TLR4 protein expression was located in the cancer cells or normal epithelial cells in the cytoplasm, where TLR4 protein expression was also observed occasionally in the nucleus (Lizundia *et al.,* 2008). Immunostaining scoring was performed based on the number of positively stained cells and the Immunostaining intensity after tissue sectioning and Immunostaining.

This study demonstrated that 24 out of 30(80%) malignant cases were positive for TLR4 expression. While 6 patients of 30(20 %) malignant cases for Score 1 which consider as TLR4negative. TLR4 immunohistochemistry scoring of 73 %(33/45) of patient (malignant and benign) have strong positive TLR4sugests that

TLR4 genes are over-producing the TLR4 protein and that those cells are growing rapidly. Breast cancers tend to be much more aggressive and fast growing. Negative expression (score 0 & 1+) of TLR4 was observed 6(20%) out of 30 patient with malignant breast tumor and 6 (40%) out of 15 patient with benign breast tumor .moderate expression (score 2+) of TLR4 appear in 9 (30%) of breast cancer patient and 8(53.33%) of benign breast tumor. 15(50%) of malignant sample showed high staining of tumor breast cell (score 3+) while in benign tumor patients 1(6.67%) as showed score 3+ TLR4 expression . the differences of this study was significant.as show in figure (3-22,23 and 24) and table (3-7).

Table (3-7) Distribution of sample study according to immunohistochemical staining for breast tumor patients (malignant and benign).

Score	Patients		Benign		Chi-square value (χ^2)
	No.	%	No.	%	
Score 0	1	3.33	2	13.33	**4.638 ***
Score 1	5	16.67	4	26.67	**4.638 ***
Score 2	9	30.00	8	53.33	**8.712 ****
Score 3	15	50.00	1	6.67	**10.793 ****
Total	30	100 %	15	100 %	---
Chi-square value (χ^2)	**12.046 ****		**12.392 ****		---
*** (P<0.05), ** (P<0.01).**					

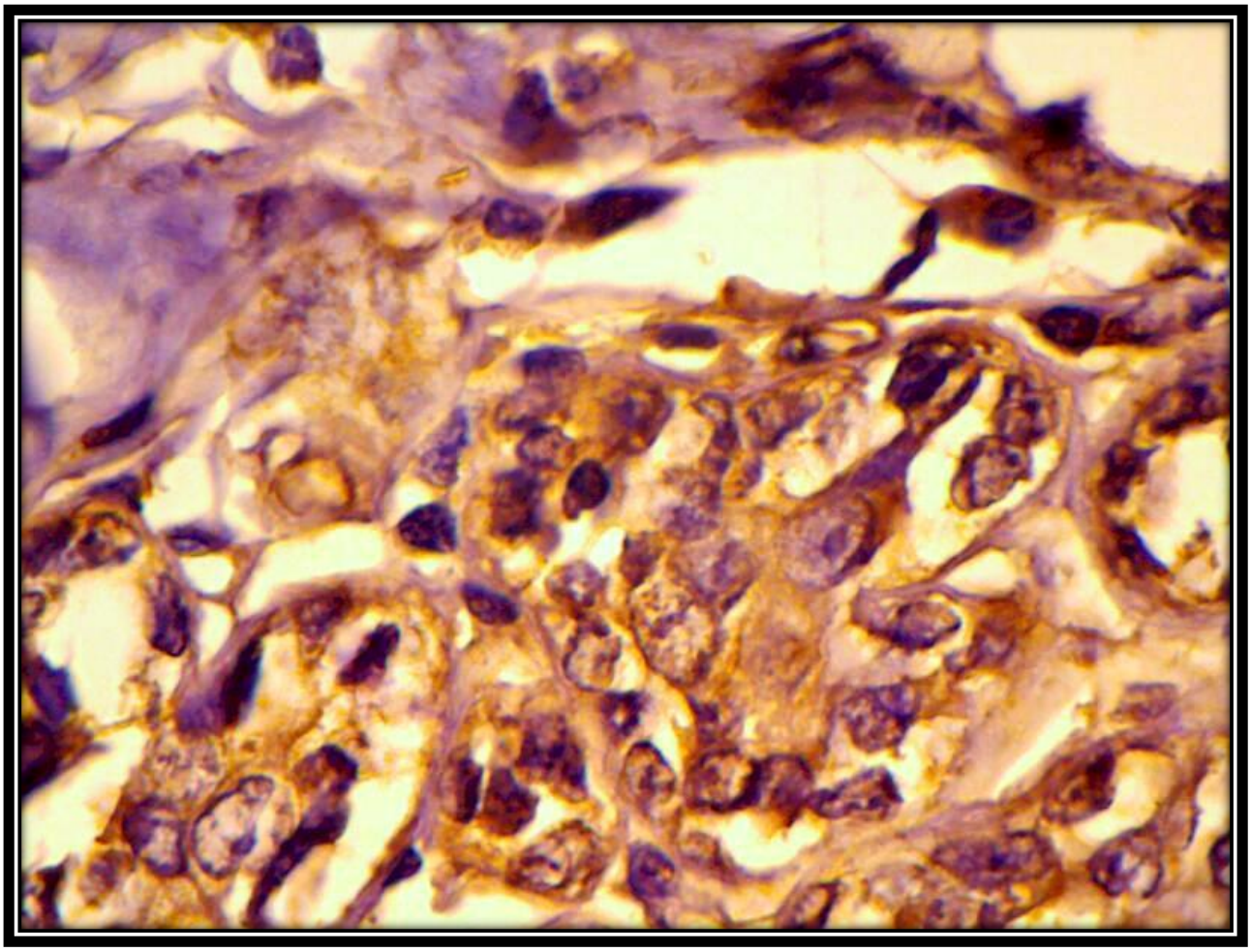


Figure (3-21): Immunohistochemical Expression of TLR4, infiltrative ductal carcinoma, score 2+. (X40).

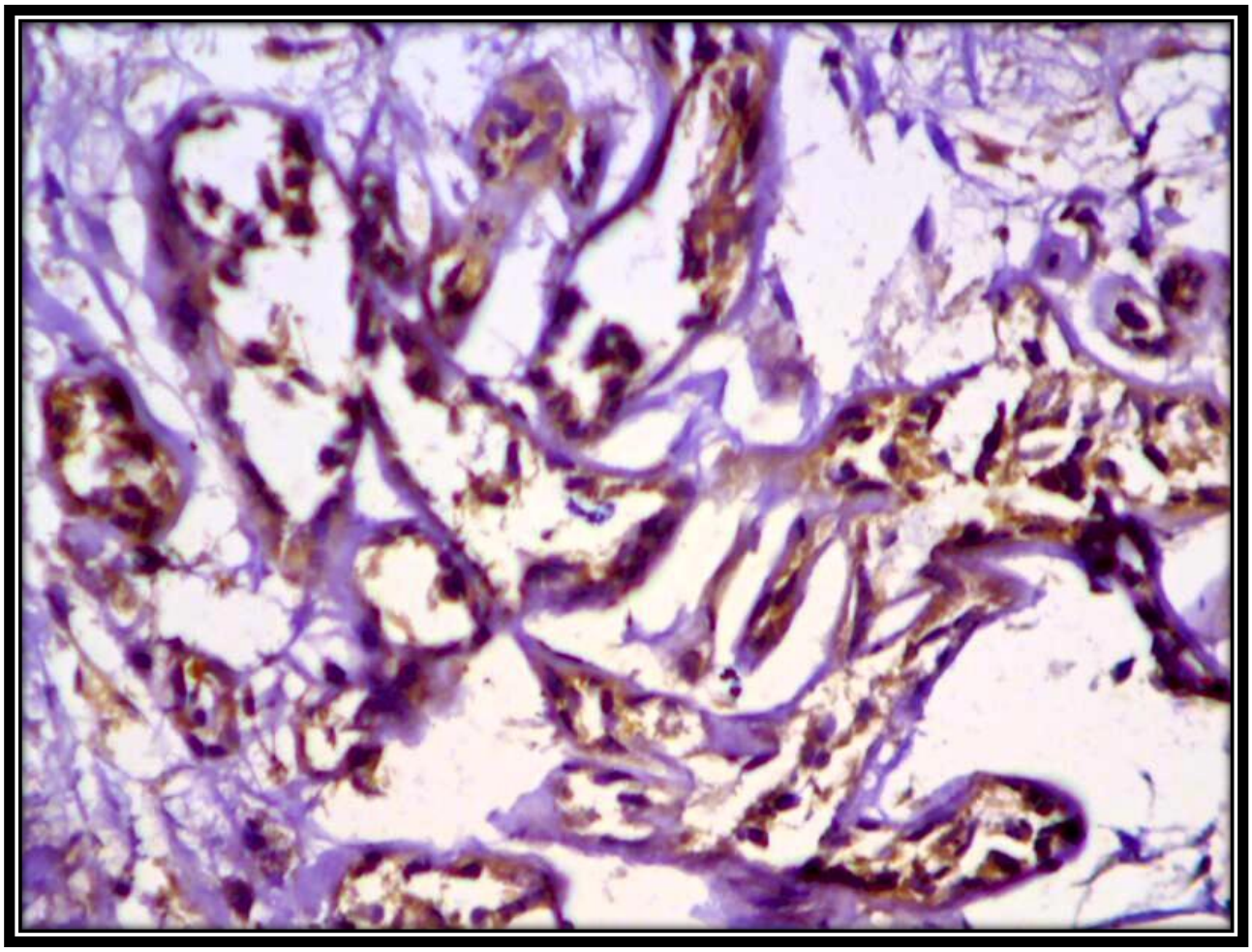


Figure (3-22) Immunohistochemical Expression of TLR4, infiltrative ductal carcinoma, score 3+ (X40).

Similar finding were recorded by Ng *et al*.,2011 in their suggestion that TLR4 overexpression by malignant breast tumor may be indicative of resistance to apoptosis mechanism, and Yuan *et al.*,2013 in their study of TLR-4 and breast carcinoma and as well, D'Adhemar *et al*.,2014 in pointed out the role of TLR4 expression in reducing overall survival (D'Adhemar *et al*.,2014), yet different results were obtained by Wang *et al.*,2014; Stopiglia *et al*.,2015 in decreasing TLR expression in tumor cells.

The outcome of these studies and the present study in term of increase or decrease TLR-2&TLR4 expression has been proposed to regard TLR4 could leads to cellular defense mechanisms up-regulation through recruitment of leukocytes and increase of vascular permeability, to be followed by lysis of tumor cell by both natural killer (NK) and cytotoxic T cell or provide microenvironment that is necessary for tumor cells to evade the immune response and proliferate (Drexler and Foxwell, 2010). As TLRs promotes carcinogenesis by up-regulation of NF-κB cascade as well as, by production of anti-apoptotic proteins, which were explained by the difference in intensity and nature of the inflammatory response with chemokines expressed by tumor cells and host cells regulating the migration of different leukocyte type as, macrophages, T cells, NKcells and dendritic cells (Rakoff and Medzhitov2009), and the defense cell proportion within the tumor will determine the immune profile at the tumor site(Srikrishna and Freeze,2009). These immune cells impair the anti-tumor function by release more proangiogenic factors, proinflammatory cytokines and growth factors, also increased functional DNA repair and up-regulation of DNA repair genes (Harberts and Gaspari, 2013)

Conclusions

1- The women at the age more than 40 which included in this study were more susceptible to malignant breast cancer incidence.

2- DNA was successfully extracted from FFPE sections and used for TLR4(exon 2, 3, 4) and MYD88(exon 1 and 5) studies

3- The present study revealed that mutations in TLR4 gene (exons 3) detected successfully in malignant breast cancer while to mutations in MYD88 gene (exons 1) of malignant breast cancer patients.

4- The positive expressions of tissues of malignant and benign breast tumor were observed in 80% and 60 % respectively.

Recommendations

1- It is highly recommended to search for new prognostic and predictive early markers to facilitate the early diagnosis of breast cancer.
2- The gene expression, mutation frequency of TLR4 and MYD88 genes in breast cancer require further investigations to determine their relation to breast cancer,
3- Using a new molecular technique for breast cancer diagnosis like DNA Microarrays to study the genes profile expression, Oncotype testing, Micro-RNA and quantitative Real Time PCR.
4- Getting fresh samples is better for molecular studies like PCR.
5- These recommendations would be better evaluated with larger numbers of breast cancer patients and controls, and interpreting the results in the light of histopathological types, grade and stage of tumor, as well as other demographic parameters; for instance, family history of breast cancer or other cancer, menopause, oral contraceptive use, and body mass index.

2.1 Material and Methods.

2.1.1 Equipment and Instruments

Various Equipment and Instruments used in this study were listed in table (2-1)

Table (2-1) Equipment and Instruments used in this study

Apparatus	Company / country
Autoclave	HIRAYAMA
Cool box	China
Deep freze	Japan
Distillater	Gallenkamp / England
Electrophoresis equipment	JUNY1-JY200C
Eppendorf tube (1.5 ml , 0.5 ml)	Gallenkamp / England
Gel –documentation system	Bio-Red / U.S.A
Laminar air flow hood	Techne / UK
Microcentrifuge	Eppendorf / Germany
Microatom	UK
Nanodrop spectrophotometer	Techne / U.K.
Oven	Sanyo / Japan
PCR thermal cycler	Multi Gene /Canada
PH meter	Martini / Japan
Refrigerator	Turkey
Sensitive balance	Mettlev / Switzerland
Ultra violet transiluminator	Consort / Germany
Vortex	Griffin / Germany
Water bath	Memmert / Germany

2.1.2. Chemicals and Solutions

various chemicals and solutions which were used in the study listed in table (2-2).

Table (2-2) chemicals and solutions used in this study

Chemicals	Company/country
Absolute ethanol	BDH / England
Agarose	Biobasic/Canada
Buffer TBE 10X	Conda / Spain
DNA ladder (2000bp)	Bioneer/Korea
Ethidium bromide	Promega /USA
Free nuclease distil water	Promega /USA
Isopropanol	Fluka / Switzerland
Loading dye	Bioneer/Korea
Proteinase K	Promega ./USA
Mastermix	Promega ./USA
Primers	Bioneer/Korea

2.1.3. Kits

No.	Kit Name	Company	Origin
1	g SYNC™ DNA Mini Kit /whol blood	Geneaid	Korea
2	FavorPrep Tissue Genomic DNA	Favorgen	Taiwan
3	SuperImmunoHistoMountTM	BioVision	US

2. 1.3.1 DNA Extraction Kit (from paraffin embedded tissues)

FavorPrep Tissue GenomicDNA Extraction Mini Kit favorgen / Taiwan which were used in the study listed in table (2-3).

Table (2-3): Contents of DNA Extraction Kit(from paraffin embedded tissue)

Componets	K-3032 (100 reaction)
FATG 1 Buffe	30 ml
FATG 2 Buffer	30 ml
W1 Buffer	44 ml
Wash Buffer	20 ml
Elution Buffer	30 ml
Proteinase K	11 mgx2
Collection Tube	200 pcs
Elution Tube	100 pcs
FATG Mini Column	100 pcs
Micropestle	100 pcs

Figure (2-1) : FavorPrep Tissue GenomicDNA Extraction Mini Kit.

2.1.3.2. DNA Extraction Kit (from blood)

Genomic DNA Extraction by gSYNC™ DNA Mini Kit Whole Blood Protocol/Geneaid/ Korea which were used in the study listed in table (2-4).

Table (2-4): Contents of DNA Extraction Kit from whole Blood.

Contents
GST Buffer
GSB Buffer
W1 Buffer
Wash Buffer
Elution Buffer
Proteinase K
GD Column
2ml Collection Tube

2.1.3.3. Immunohistochimsrty kit:-

Ready-to-use IHC/ICC kit (Biotin free) from bioVision/USA which were used in the study listed in table (2-5) **.**

Table (2-5): Contents of immunohistochimsrty kit

Components	K405-50
Peroxidase Block (H2O2)	5 ml
Protein Blocking solution	5 ml
Primary Ab dilution buffer	8 ml
HRP-anti-Mouse, Rat & Rabbit Polymer	5 ml
Reagent BS (buffer & substrate)	5 ml
Reagent C (conc. DAB chromogen)	1ml

2.1. 4. Solutions used in DNA Extraction From Tissue

- **Xylene**

It´s ring aromatic organic solvent that is used to dissolve paraffin in the FFPE samples **.**It is ready to be used **(**FLUKA, Germany). Every time, 500µl is added to 25µg of tissue in 1.5µl eppendorf tube to remove the wax .

- **Beta-mercaptoethanol (ß-ME)**

Is a reducing agent that will irreversibly denature RNases by reducing disulfide bonds and destroying the native conformation required for enzyme functionality (Knight, 2004). 200µl is added to 25µg of tissue in 1.5µl eppendorf tube to remove the wax .

2.1.5. Solutions used in Agarose Gel Electrophoresis

- **Tris -borate (TBE) Buffer**

(0.89M Tris-Base ; 0.88M Boric acid ; 20mM EDTA ,PH 8.0)

To prepare 10X TBE solutions , the component used as following : 108gm of Tris-Base , 55gm of Boric acid , 40ml of 0.5M EDTA (pH = 8.0) in an appropriate amount of D.W , pH was adjusted to 7.8 and volume completed to 1 liter with D.W . The solution was sterilized by autoclave and stored at room temperature (Sambrook *et al.* ,1989).

- **Ethidiume Bromide Dye (10 mg / ml)**

This was prepared by dissolving 1gm of Ethidium Bromide in 100 ml of a sterile D.W, and the bottle was kept in the dark (Maniatis *et al*. ,1982). Ethidium is a powerful mutagen ;so the gloves and the mask should be worn in all the steps during the work.

2.2. study subject.

The study involved 70 women with breast tumor, who were distributed into two groups of malignant and begnin group. Malignant tumor group included 50 patients whose age range was between 30 and 65 years (52.13 ± 9.61year), while benign tumor group that included 20 patients range of age was between 21- 60 years (38.58 ± 7.95 year).

The patients were referred to the AL- Emamain AL- Kadhemaian teaching Hospital in Baghdad during the period April – November 2013. all samples were diagnosis by specialist physician as a breast tumor females that don’t treated with any anticancer therapy. Furthermore, the patients were also followed-up after the surgical operation to define the histopathological classification of breast tumor. With respect to controls were 25 individuals that enrolled in this study , their age range was 18-64 year (41.28 ± 13.10 year). They were ethnically matched with breast tumor patients (Iraqi Arabs).

2.2.1 Blood Samples Collection.

The blood taken from 25 healthy individuals as control group. Six ml of venous blood collected in a 10ml disposable syringe then transferred to EDTA tube for DNA extraction and transported in cool box to the laboratory of Molecular department of Biotechnology Research Centre /AL-Nahrain University. Blood samples were kep at -20c to be analysid when collected.

2.2.2 Paraffin Empbeded Tissues Samples Collection

Fifteen samples of Formalin-Fixed Paraffin Embedded Tissue (FFPE) from malignant BT patients and 20 from bening BT patients were collected from AL- Emamain AL- Kadhemaian teaching Hospital.

2.3. Method

2.3.1. DNA extraction from blood and paraffin empbeded tissues samples

DNA was extracted from blood sample and paraffin embedded tissues in the Molecular department of Biotechnology Research Centre /AL-Nahrain University. DNA was extracted from the samples by using Genomic DNA Extraction Kit geneaid / Taiwan and FavorPrep Tissue GenomicDNA Extraction Mini Kit favorgen / Taiwan (for paraffin embedded tissues) (table 2-3 and 2-4**).**

2.3.1.1 DNA Extraction from Formalin-Fixed Paraffin Embedded Tissue (FFPE).

Formalin fixation and paraffin embedding (FFPE) is a commonly used method for archiving tissue specimens .The ability to extract DNA from these samples provides the potential for correlating disease state and tissue morphology with genotype **(**Chaux *et al*., 2012) .

Extraction of DNA from FFPE tissue historically has been a challenge because the formalin fixation process results in cross-linking between protein and DNA, as well as between different strands of DNA . The non optimal preservation of genomic DNA in FFPE complicates its use in many standard downstream analysis applications(Lin, 2009).

In this study , Method that had been used for DNA extraction was FavoPrep DNA Mini Kit (FavorGen), (Taiwan).

1. Paraffin-embedded tissue sample was Cut up to 25 mg to a microcentrifuge tube .
2. added 1 ml xylene , mixed well and incubated at room temperature for 30 min. Centrifuged at full speed for 5 min. The supernatant was Remove by pipetting. 1 ml ethanol (96 ~ 100%) Add to the deparaffined tissue, mixed gently by vortexing.
3. Centrigued at full speed for 5 min. the supernatant was Removed by pipetting.
4. Added 1 ml ethanol (96 ~ 100%) to the deparaffined tissue, mixed gently by vortexing.
5. Centrifuged at full speed for 5 min. The supernatant was Added by pipetting.
6.Then Incubated at 37 C for 10 min to evaporate ethanol residue.
7. added 200 µl FATG1 Buffer and homogenized the tissue sample more completely with micropestle.
8. added 20µl Proteinase K (10mg/ml) to the sample mixture. Mix thoroughly by vortexing.
9. Incubated at 60 C until the tissue is lysed completely. Vortexed every 10-15 min during incubation.
10.The tube was spainned to remove drops from the inside of the lid.
11. added 200µl FATG2 Buffer to the sample mixture and mixed thoroughly by pulse-vortexing and incubated at 70 C for 10 min.

12. The tube spanned prefly to remove drops from the inside of the lid.
13. added 200 µl ethanol (96 ~ 100%) to the sample and Mixed thoroughly by pulse-vortexing.
14. The tube was spanned prefly to remove drops from the inside of the lid.
15. FATG Mini Column was placed in a Collection Tube Then transfered the sample mixture carefully to FATG Column thenCentrifuged for 1 min and the flow- through was discarded then placed FATG Column to a new Collection Tube.
16. FATG Column was washed with 500µl W1 Buffer by centrifuge for 1 min then discard the flow-through. Make sure that ethanol has been added into W1 Buffer when first open.
17. Washed FATG Column with 750µl Wash Buffer by centrifuge for 1 min then discarded the flow-through.
18.Then Centrifuged for an additional 3 min to dry the column
20. FATG Column was placed to Elution Tube.Then Added 50µl Elution Buffer or ddHO (pH 7.5 ~ 8.5) to the membrane center of FATG Column. Stand FATG Column for 3 min.
21. then Centrifuged for 2 min to elute total DNA.
22. Total DNA was stored at 4 C or -20 C

2.3.2. Isolation of DNA from whole blood.

The Wizarad Genomic DNA purification Kit provides a simple, solution-based methods for isolation of DNA from whit blood celles according to Genaid mini kit.

Red Blood Cell Lysis:

1. transferred 900 µl of RBC Lysis Buffer and 300 µl of whole blood into a 1.5 ml microcentrifuge tube then mixed by inverting.
2. Incubated for 5 minutes at room temperature
3. centrifuged at 14,000 rpm for 5 minutes to form a leukocyte (buffy coot) pellet. The supernatant was carfully removed , retaining approximately 50 µl of residual buffer and leukocyte pellet. The tube until the leukocyte pellet is completely resuspended in the residual buffer.

Buffer lysis

1. added 300 µl of Cell Lysis Buffer to the tube then mixed by vortex.
2. Incubated at 60ºC for at least 10 minutes to ensure the sample lysate is clear and homogenous. During incubation, the tube was inverted every 3 minutes.

Protein Removal

1.added 100 µl of Protein Removal Buffer to the sample lysate then vortexed immediately for 10 seconds.

2.Centrifuged at 14000 rpm for 3 minutes to form a tight, dark brown, protein pellet.

DNA Precipitation:

1.The supernatant was transfered to a clean 1.5 ml microcentrifuge tube

2. added 300 µl of isopropanol and mixed well by gently inverting 20 times.

3.Centrifuged at 14,000 x g for 5 minutes then carefully discard the supernatant was carfully discarded

4.add 300 µl of 70% ethanol added to wash the pellet.

5.Centrifuged at 14000 rpm for 3 minutes then the supernatant was carefully discarded and the pellet was air-dried for 10 minutes.

DNA Rehydration:

1.added 100 µl of DNA Hydration Buffer then gently vortexed for 10 seconds.

2.Incubated at 60ºC for 5minutes to dissolve the DNA pellet. During incubation, the bottom of the tube was tapped to promote DNA rehydration.

2.4. Measurement of DNA Concentration and Purity.

Concentration and purity of extracted DNA were measured by the nanodrop spectrophotometer. The principle of measurement DNA concentration by nanodrop depends upon the amount of UV irradiation absorbed by the nitrogen bases composing the DNA. 2µl of DNA was loaded to the lens of the nanodrop and measured on 260/280 nm wave length, the result appeared on the laptop screen that attached to the nanodrop .The nanodrop lens was cleaned by distilled water (D.W) and cotton swap after each sample, the other samples were measured consequently. The DNA concentration from FFPE samples was ranged btween 50 – 100 ng/ µl while the DNA concentration from blood samples was higher and ranged between 100-250 ng/ µl.

2.5. Agarose gel electrophoresis

2.5.1. Reagents used in agarose gel electrophoresis (Vogelsein and Gillespie , 1979)

- Agarose
- 10 X TBE Buffer.
- Bromophenol blue (Loading dye) .

- DNA marker.
- Ethidium bromide (10mg / ml).

2.5.2 Preparation of agarose gel electrophoresis

1. Preparation of 1 Liter of TBE Buffer (1X)(Younan**,** 2010**):**

100mL of 10X TBE + 900 mL of D.W

2. Agarose gel was prepared according to Maniatis *et al*., 1982.

●A 100 ml of 10 X TBE was placed in a beaker.

●Then a 0.8 g or 2g agarose was added to the buffer (as required) to prepare 0.8% and 2% agarose gel respectively.

●(the same concentrations was used in case of low melting agarose gel).

●The solution was heated to boiling (using heating stirrer)

●Then the solution was allowed to cool down. Then a 10 µL of Ethidium Bromide solution was added.

3. Casting of the horizontal agarose gel:

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

●The agarose solution was dropped into the gel tray after both edges were sealed with tapes and the agarose was allowed to cool at room temperature for 30 minutes.

●The comb was carefully removed and the gel replaced in electrophoresis chamber.

●The chamber was filled with an appropriate amount of TBE electrophoresis buffer so that it covers 1-2 mm over the surface of the gel.

●Ten µl of the 2000 bp DNA Ladder

●Ten µl of the sample was applied to each lane of the gel. The lid was placed on electrophoresis t - ank.

●The cathode was connected to one side of the unit and the anode to the other side.

- It was run for 10 mim at 100v then at 70v for 1 hour.
- The DNA was observed by UV transiluminator (λ=305nm).

2.6. Amplification of DNA by PCR technique.

PCR reaction performed using the following

1. Specific primers and their prepration

All the primers and primers with their sequences used in this study and designed by Bioneer company (Korea).the details of these primers which including sequences are presented in table (2-6) which provided in lyophilized form and dissolved in sterile distilled water to have the final concentration of 10 pmol/µl.

Table(2-6): A. Sequences of the TLR4 primers used.

No.of primer	Name	Oligonucleotides	Product length	Sequence (5'-3')
1	TLRX1F	Forward	**853 bp**	TATTGCACAGACTTGCGGGT
	TLRX1R	Reverse		CTTCGAGACTGGACAAGCCA
2	TLRX2F	Forward	**967 bp**	AGAGGGCCTGTGCAATTTGA
	TLRX2R	Reverse		CTGCCTCTGGTCCTTGATCC
3	TLRX3F	Forward	**919 bp**	GGATCAAGGACCAGAGGCAG
	TLRX3R	Reverse		ATTCCCTCTGCACTGGAAGC
4	TLRX4F	Forward	**720 bp**	TGCAAGATGCCCCTTCCATT
	TLRX4R	Reverse		TCAAGGAGCATTGCCCAACA

Table(2-6):B. Sequences of theMYD88 primers used.

No. of primers	Name	Oligonucleotides	Product length	Sequence (5'-3')
1	MYDXF1	Forward	**606 bp**	GATTCCTACTTCTTCTTACGCCCC
	MYDXR1	Reverse		CGGAAAGTCAGCCTCCTCAC
2	MYDXF1	Forward	**877bp**	GCAGTGCAAGTCCCCAAGA
	MYDXR1	Reverse		ACAGGTGCATGGCACAGCTAAA

2. Green Master Mix from Bioneer / Korea

PCR master mix is a powerful and ready to use PCR reagent optimized for perfect PCR amplification. The main advantages of this mix are used for reduction time in PCR setup, also it is a powerful technology for convenient and easy performance of DNA amplification. (Xu *et al* .,2011). Table (2.7) shows the PreMix contents.

Table (2-7): PCR reaction components for Green Master Mix.

NO.	Component	Quality/ concentration
1	Taq polymerase	2.5µ
2	dNTP (dATP, dCTP, dGTP, dTTP)	250 µM
3	Tris – HCL (ph 9.0)	10 mM
4	KCL	30mM
5	Mgcl	1.5mM

2.7. Polymerase Chain Reaction (PCR).

PCR technique was used to identify and amplify TLR4 and MYD88 genes, by using the primers that designed by Bioneer/Korea (table: 2.6). The primers were lyophilized, then dissolved in a free ddH2O to give a final concentration of 100 pmol/µl (as a stock solution) and kept as a stock in -20°C.

The total volume of PCR reaction was 25µl, the reaction components are described in table (2-8), after preparing the reaction volume in PCR tube (200µl) the mixture was spin down, and then PCR tube placed in PCR thermal cycler and PCR amplification was started according to the program described in table (2-9, 2-10) for 2:45 h (Li *et al*., 2011).

Table (2-8): PCR reaction components **volume**

Material con.	Final con.	Volumw for 1 tube/ µl
D.W	-	8.5
Master mix	1X	12.5
Primer (forward) 10 pmol/ µl	1 **µl**	1
Primer (reverse) 10 pmol/ µl	1 **µl**	1
DNA	**-**	2 **µl**
Total reaction volume		25

2.8. PCR Protocol.

For PCR amplification of TLR4 gene (exon 1, 2, 3, 4) the following PCR protocols was followed :

Table (2-9) PCR Amplification using primer 1,4 (exon 1, 4)

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94^0C for 3 minutes
Denaturation	35cycle	94^0C for 1 min
Annealing		60.6^0C for 1 min
Extension		72^0C for 2 min
Final Extension	1 cycle	72^0 C for 10 min.

Table (2-10) PCR Amplification using primer 2 (exon 2, 3)

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94^0C for 3 minutes
Denaturation	35cycle	94^0C for 1 min
Annealing		60.5^0C for 1 min
Extension		72^0C for 2 min
Final Extension	1 cycle	72^0 C for 10 min.

For PCR amplification of exon (1,5) of MYD88 gene the following PCR protocols was followed :

Table (2-11) PCR Amplification using specific primer

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94^0C for 3 minute
Denaturation	35cycle	94^0C for 1 min
Annealing		61^0C for 1 min
Extension		72^0C for 1 min
Final Extension	1 cycle	72^0 C for 10 min.

2.9. DNA Loading and Electrophoresis

The PCR product was separated on 2% agarose gel at 100 volt for 10 min in 1X TBE buffer by taking 10µl from each sample, seven µl of the 25 bp DNA ladder was used as a marker.

Agarose gels were stained with ethidium bromide 0.5mg/ml for 20-30 minutes. DNA band was visualized by electrophoresis and captured by gel documentation system to document the observed bands.

PCR products (5 µl) were loaded carefully into the individual wells of the gel. There were no need to mix PCR products with loading dye because the Blue Master Mix reaction buffer is proprietary buffer containing a compound that increases sample density, and blue dyes, which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis, and Electrical power was 5 volt/ cm for (1-2 h) afterwards the DNA moved from cathode (-) to anode (+) poles due to the negative charge of the DNA. The Ethidium Bromide stained bands in the gel were visualized using UV transiluminator at (302-320) nm

wavelength and photographed by used gel documentation system to document the observed bands (Sambrook *et al*., 1989).

2.10. Measurement of DNA concentration before sequencing.

Before sequencing, DNA concentration of the TLR4 gene (exon 1, 2, 3, 4) and MYD88 gene (exon١, 5) PCR products from ٥٠ malignant BT samples and 20 benign patients and 25 healthy was measured using nanodrop (techne UK). All products gave concentration more than 100n g/µl which is the least concentration required for DNA to be sequenced.

2.11. DNA sequencing .

Polymerase chain reaction products TLR4 exon 1, 2, 3, 4 and MYD88 gene exon(١,5) of 40. The obtained sequences were aligned using NCBI software with normal sequence from NCBI GenBank and examined of the presence of SNPs.

2.12. Immunohistochemistry method.

1. Tissue sections was Deparafinize and hydrate through xylene and graded alcohols.

- Xylene 5 minutes (twice).
- 2- 99 % ethanol 5 minutes (three time).
- 3- 95 % ethanol 5 minutes.
- 70 % ethanol 5 minutes.
- 50 % ethanol 5 minutes.
- Distilled Water.

2. sections were washed 2-3 times with distilled water.

3. sections' was Incubate with Peroxidase Block for 5-10 minutes at room temp. Wash with distilled water 3 times.

4. slide washed with PBS or Tris saline buffer (with 0.02-0.05% nonionic detergent, Triton X100, Tween 20 or NP-40) 3 times.

5. sections incubated in Protein blocking solution , for 5-10 minutes at RT.

6. sections was incubated with primary antibody for 20-30 minutes at RT. The primary antibody dilution buffer supplied can also be used as a negative control.

7. slide was washed with PBS 5-7 times. Incubate with One-Step HRP polymer for 20-30 minutes at RT.

8. slide was washed 5-7 times with PBS or TBS preferably with 0.025-0.05% non-ionic detergent (Triton x-100 or Tween- 20 or NP-40) without sodium azide.

9. slide wased with distilled for 2-3times.

10. In a test tube, add 1 ml of Reagent BS & 50 µl of Reagent C (DAB chromogen) were added . Mixed added well**.** few drops of ready was added to use DAB reagent on tissue slides & wait for 6-10 minutes at RT.

12. slide wased 5-7 times with PBS, followed by rinsing with distilled water.

13. slides were ncubate with counterstain compatible with DAB for 30-60 sec. We prefer hematoxylin.

14. slide were washed with distilled water.

15. slide were Mount with appropriate mounting medium e.g. SuperImmunoHistoMountTM

2.13. Scoring of the immunohistochemical staining for TLR4 overexpression (Hammond *et al*., 2011):

Score 0: Negative ,No staining is observed or membrane staining is observed in less than 10% of the tumor cells.

Score +1: Negative, A faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane .

Score +2: Weakly positive, A weak to moderate complete membrane staining is observed in more than 10% of the tumor cells.

Score +3: Strongly positive, A strong complete membrane staining is observed in more than 30% (formerly 10%) of the tumor cells.

Table (2-12): Nottingham scoring system

Grade	Combined Score	Description
I	3–5	Low-grade (well-differentiated) tumours that do not appear to be growing quickly and are less likely to spread
II	6–7	Intermediate-grade (moderately differentiated) tumours that have features between grade 1 and 3
III	8–9	High-grade (poorly differentiated) tumours that tend to grow faster and are more likely to spread

2.14.Statistical Analysis.

The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters . Chi-square test was used to significant compare between percentage in this study.

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1.1 Introduction

Breast cancer is one of the most common malignancies affecting women worldwide with a mortality rate of more than one million per year (Siegel *et al*., 2012). In 2015, nearly 1.7 million new cases of breast cancer diagnosed. Although breast cancer patients have prolonged disease-free survival and overall survival or even been cured after receiving systemic therapy. There are still some patients died of the disease due to development of chemo résistance and lack of therapeutic targets (Shaheenah, 2015).

In Iraq, Breast cancer is the most frequent cancer among women and according to the latest Iraqi Cancer Registry, it account for approximately one third of the registered female cancers in Iraq, indicated that the breast cancer is the leading cancer site among females (Iraqi national cancer research center , 2013). According to the data during the period between 2000 to 2009 the total of 23,792 incidence breast cancer cases were registered among female aged ≥ 15 years, represented 33.8 of all cancers(Al-Hashimi *et al* ., 2014).

Different breast cancer-associated risk factors have been suggested to play a role in pathogensid of breast cancer for instance, age, menarche, menopause, parity, breastfeeding, use of exogenous hormones or oral contraceptive, obesity, lack of exercise, diet, smoking, alcohol consumption and family history of breast cancer or other cancers. Therefore, breast cancer is considered clinically heterogeneous and complex disease, which involves a wide variety of pathological conditions and a range of clinical behavior. This heterogeneity is strictly linked to individuals and tumors of genetic variability; accordingly, it is widely accepted that genetic abnormalities accumulation contributes to the acquisition of increasingly invasive breast cancer (Cavallaro *et al*., 2012).

Several study have been made to identify genetic susceptibility for this malignancy, both through analyses of hereditary factors associated with familial risk of early-onset disease, and through gene-wide association studies (Shen *et al*.,2010). The immune system plays dual host-protective and tumor-promoting roles in breast cancer initiation and progression, by mechanisms that may shield breast cancers from immunosurveillance and enable breast cancer cells to evade immune cell induced apoptosis and produce an immunosuppressive tumor microenvironment (Jiang and Shapirob, 2013) Accordingly, breast cancer is clinically regarded as a heterogeneous and complex disease, encompassing a wide variety of pathological entities and a range of clinical behavior. This heterogeneity is strictly linked to individuals and tumors genetic variability; therefore it is now widely accepted that accumulation of genetic anomalies contributes to the acquisition of an increasingly invasive or chemo-resistant tumor phenotype (Cavallaro *et al*., 2012).

Many genes of immune system components which participate in inflammatory response are study regarding their role in BCa. Two of these genes are toll-like receptor 4 (TLR4) genes and myeloid differentiation 88 (MyD88) genes. These genes play an important biological function in pathogenesis by mediating tumor invasion and migration, escaping from immunosurveillence, promoting tumor proliferation, inhibiting apoptosis and developing chemo resistance in several type of cancer, including ovarian cancer, and prostate cancer (Wang *et al*., 2010).

Furthermore, in breast cancer, lipopolysaccharide acting on downstream signaling molecules TLR4 and MyD88 gene were regulates the growth rate of tumor cells by reducing the expression of TLR4 or MyD88 molecules (Egunsola *et al*., 2012).

Aim of study:-

1- Detecting abnormalities in MYD88 and TLR4 genes in breast cancer to Investigate molecular mechanism that involved in pathogenesis of breast cancer in an attempt to participate in the development of new treatment strategy that my improve quality of life of patient.

2- Immunohistochemical evaluation of Toll like receptor 4 expression.

1.2. Literature review.

1.2.1. The normal breast.

The breast is the tissue overlying the chest (pectoral) muscles. Women's breasts are made of specialized tissue that produces milk (glandular tissue) and fatty tissue. The amount of fat determines the size of the breast (Laura, 2016). The milk-producing part of the breast is organized into 15 to 20 lobes. Within each lobe are smaller structures, called lobules, where milk is produced. The milk travels through a network of tiny tubes called ducts, which connect together into larger ducts, and eventually exit the skin in the nipple. The dark area of skin surrounding the nipple is called the areola as show in figure (1-1). (Jansen *et al.*, 2014).

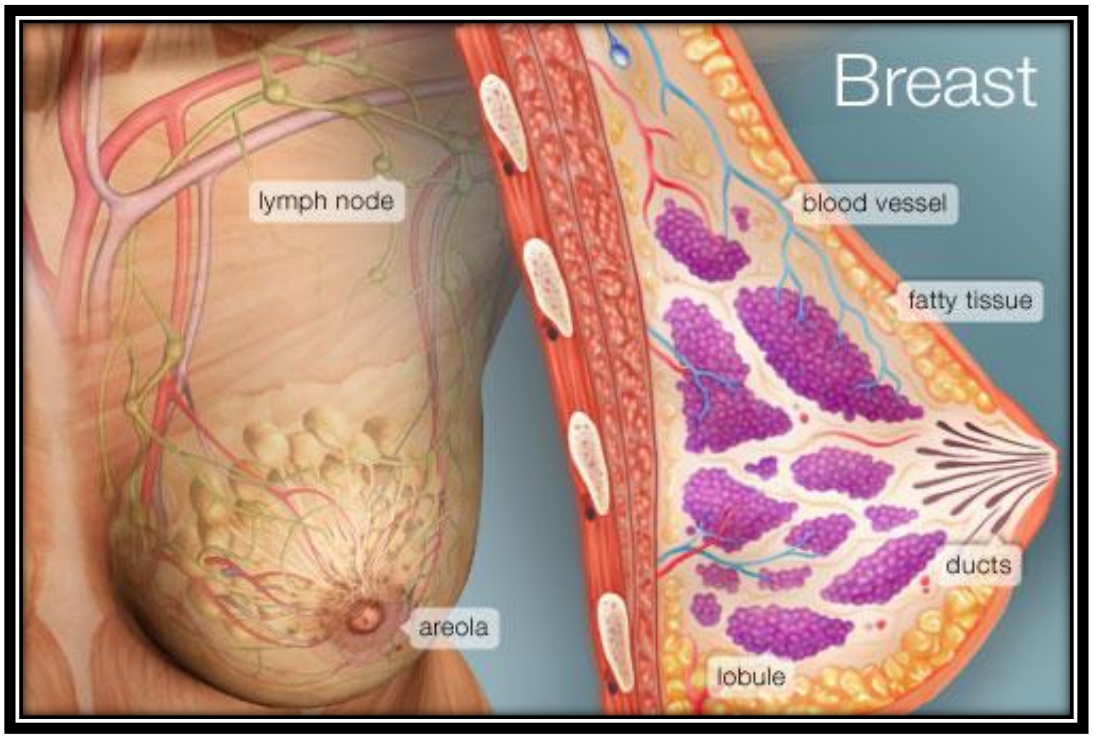

Figure (1-1): Structure of breast (Ferlay *et al.*, 2015).

1.3. Pathology of breast.

1.3.1. Benign breast conditions.

The breast is composed of two main types of tissues: glandular tissues and stromal (supporting) tissues. The glandular tissues house the milk-producing lobules and the ducts (the milk passages). The stromal tissues include fatty and fibrous connective tissue. Any changes in the glandular or stromal areas may cause symptoms of benign breast conditions (Haas *et al.*, 2005).

These changes was included an increase in the number of breast cells (hyperplasia) or the emergence of atypical breast cells (atypical hyperplasia) as show in fig (1-2). In some instances, a portion of a normal breast tissue can eventually develop into a cancerous tumor. While the appearance of atypical hyperplasia increases the risk of breast cancer, not all women with abnormal breast cells go on to develop breast cancer **(**Schindler, 2011) .The following chart summarizes the typical progression of breast tissue from "normal to cancer.

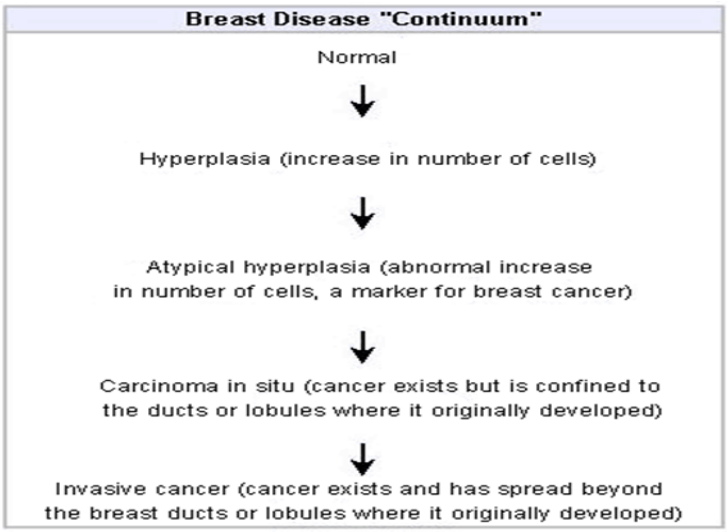

Figure (1-2) Typical progression of breast tissue from normal to cancer (Kevin *et al,* 2015).

1.3.2. Malignant Breast Tumor

Breast cancer is a malignant tumor that starts in the cells of the breast. A malignant tumor is a group of cancer cells that can invade surrounding tissues or spread (metastasize) to distant areas of the body. The disease occurs almost entirely in women (Abeloff *et al.*, 2008).

Uncontrolled cell growth gives rise to tumors which could either be benign or malignant. Benign tumors remain encapsulated whereas malignant ones have the ability to invade and form metastases. Such malignancies are described as cancers but they include various forms, each with different characteristics depending on their origin. Breast cancers derived from connective tissue such as muscle are termed sarcomas while those of epithelial lineage are carcinomas (Pecorino, 2010).

Several studies have sought to elucidate the nature of the mammary stem cell, which gives rise to the different breast cell types. A balance between cell proliferation, cell differentiation and cell death is thought to be critical for normal development (Cariati and Purushotham, 2008). For instance, conditions of up-regulated cell proliferation or down-regulated apoptosis may foster accumulation of mutations, contributing to the subsequent development of breast cancer (Russo *et al*., 2011). Advancements in the experimental transformation of human cells have shown that the disruption of certain regulatory pathways in the cell is sufficient to impart a tumorigenic phenotype to a wide variety of normal cells (Hahn and Weinberg, 2002).

Events leading to carcinogenesis were originally simplified into three distinct phases: initiation, promotion and progression (Figure 1-3). The initiation stage is thought to be rapid, involving binding and damage to DNA of a cell by a carcinogens or alterations in the stability of the normal genotype and phenotype as a

result of the local collapse in the system of intercellular processes (Steen, 2000). Cascades of mutations in these crucial genes may be synergistic and irreversible (McMurray *et al.*, 2013). At the promotion stage, there is clonal expansion of initiated cells induced by tumor promoters (e.g. mitogens) for the initiated cell. This stage is thought to be reversible and may depend on a variety of other extracellular factors, such as hormones and immunological compatibility. The progression stage represents an extension of promotion whereby continuous cell proliferation caused by promoters allows the cellular damage inflicted by initiation to be further propagated (Steen, 2000)

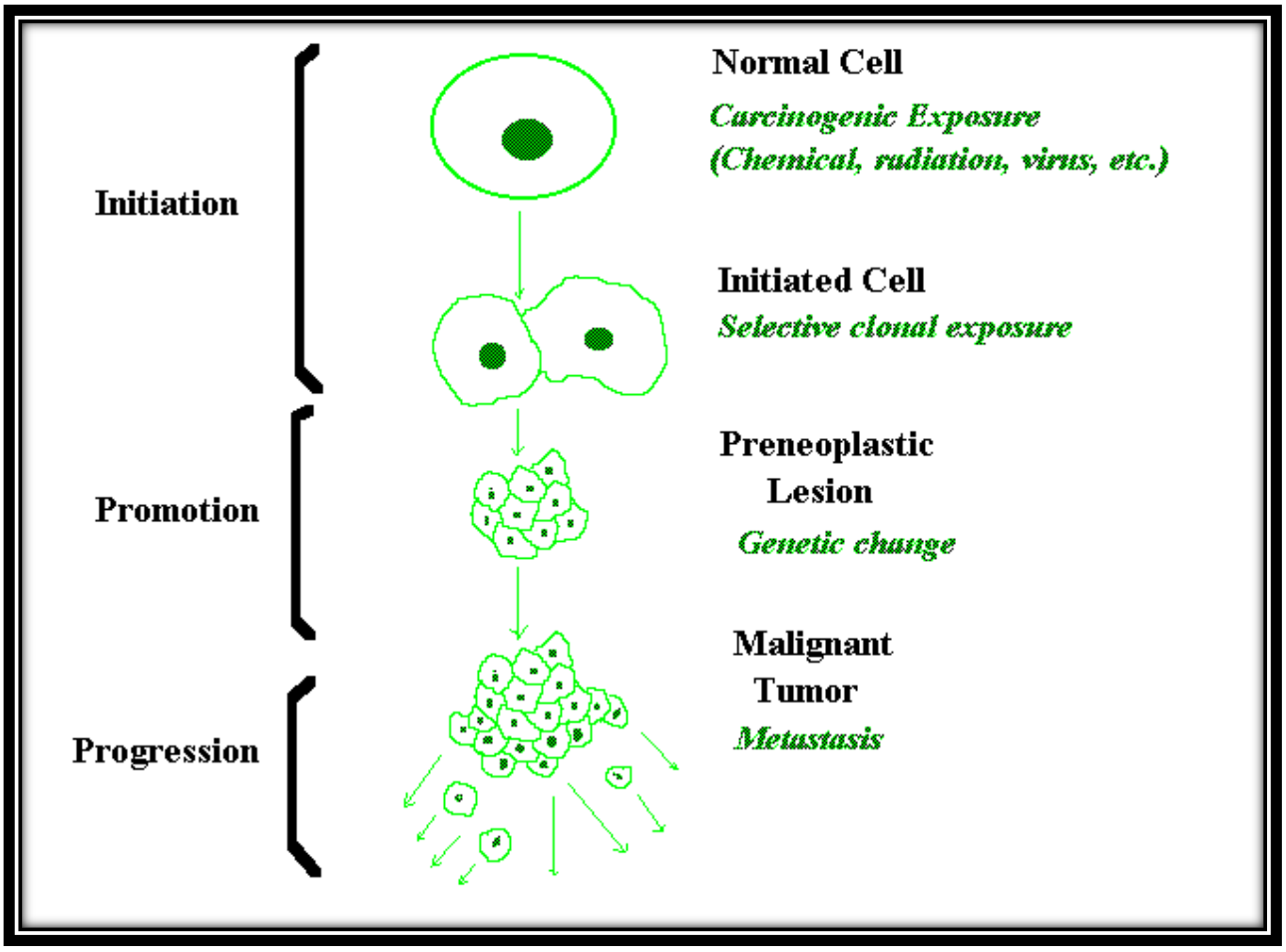

Figure (1-3): Hypothetical phases of multistep carcinogenesis (Alison, 2001).

1.4. Types of breast cancers

Several types of breast cancer exist some of them are quite rare. A single breast tumor can sometime be a combination of these types or be a mixture of invasive and in situ cancer (World Health Organization, 2014).

1.4.1. Ductal carcinoma *in situ* DCIS.

Also known as intraductal carcinoma is a pre-cancerous or non-invasive cancerous lesion of the breast (fig 1-4). Ductal carcinoma *in situ* (DCIS) is classified as Stage Zero. Rarely produces symptoms or breast lumps, and is usually detected through screening mammography. In this condition abnormal cells are found in the lining of one or more milk ducts in the breast and the abnormal cells have not moved out of the mammary duct and into any of the surrounding tissues in the breast (Welch *et al*., 2010).DCIS may sometimes become invasive cancer and spread to other tissues, it encompasses a wide spectrum of diseases ranging from low-grade lesions that are not life- threatening to high-grade (i.e. potentially highly aggressive) lesions (Breen *et al*.,2011).

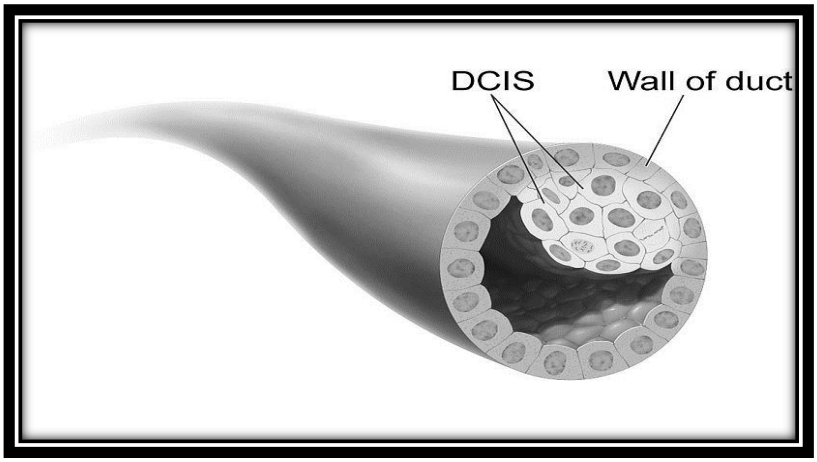

Figure (1-4): Ductal carcinoma in situ (National Cancer Institute, 2012)

1.4.2. Lobular carcinoma *in situ* (LCIS).

It is caused by unusual cells in the lobules of the breast fig (1-5). Many do not consider it cancer, but it can indicate an increased risk of future cancer. The national database registrars, however, consider it a malignancy. Unlike ductal carcinoma *in situ* (DCIS), LCIS is not associated with calcification, and is typically an incidental finding in a biopsy performed for another reason. LCIS only accounts for about 15% of the *in situ* (ductal or lobular) breast cancers (Susan, 2013).

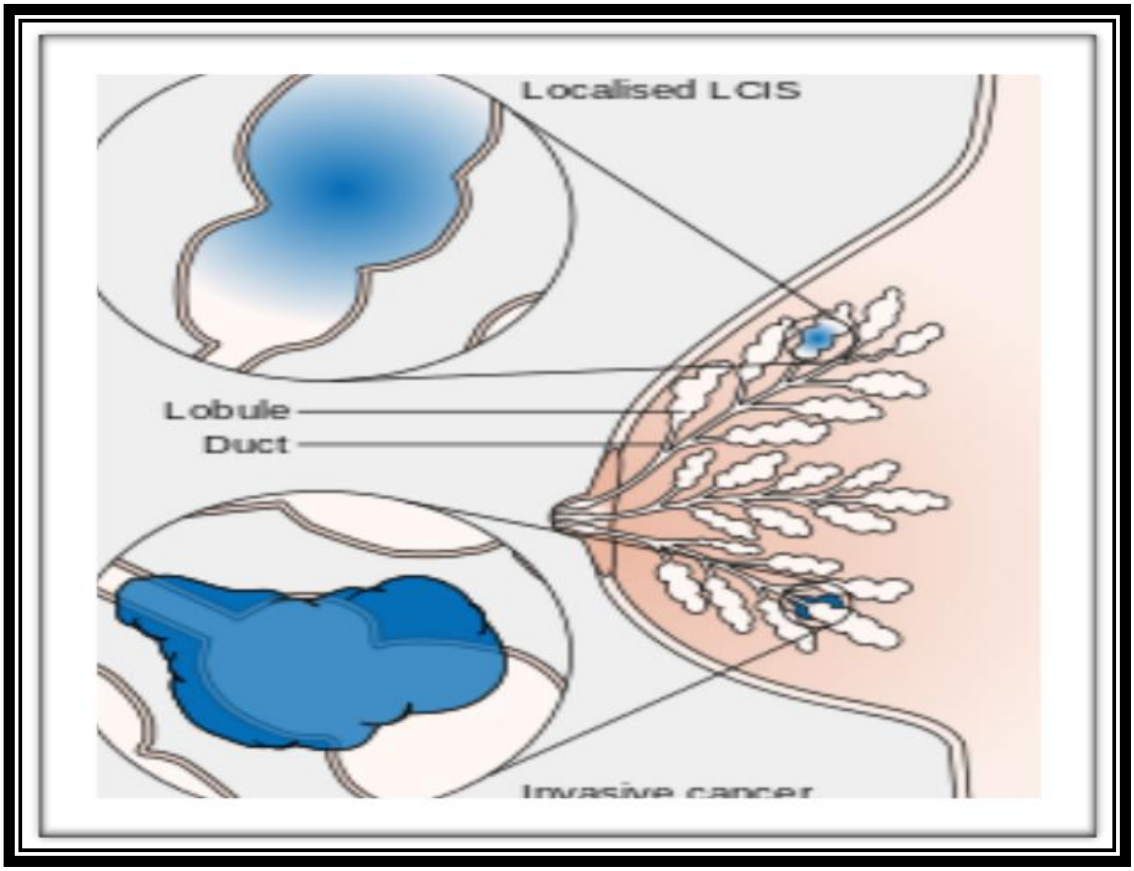

Figure (1-5): Lobular carcinoma in situ (LCIS) (simpson *et a*l.,2010)

1.4.3. Invasive (or infiltrating) ductal carcinoma IDC.

This is the most common type of breast cancer. It starts in a milk duct of the breast, breaks through the wall of the duct, and grows into the fatty tissue of the breast. At this point, it may metastasize to other parts of the body through the lymphatic system and bloodstream. About 8 of 10 invasive breast cancers are infiltrating ductal carcinomas (King and Reis-Filho, 2014).

1.3.4. Invasive (or infiltrating) lobular carcinoma ILC.

Invasive lobular carcinoma (ILC) starts in the milk-producing glands (lobules) (figure 1-6). Like IDC, it can invade other parts of the body. About 1 invasive breast cancer in 10 is an ILC. It lobular carcinoma may be harder to detect by a mammogram than invasive ductal carcinoma (Afonso *et al*., 2008).

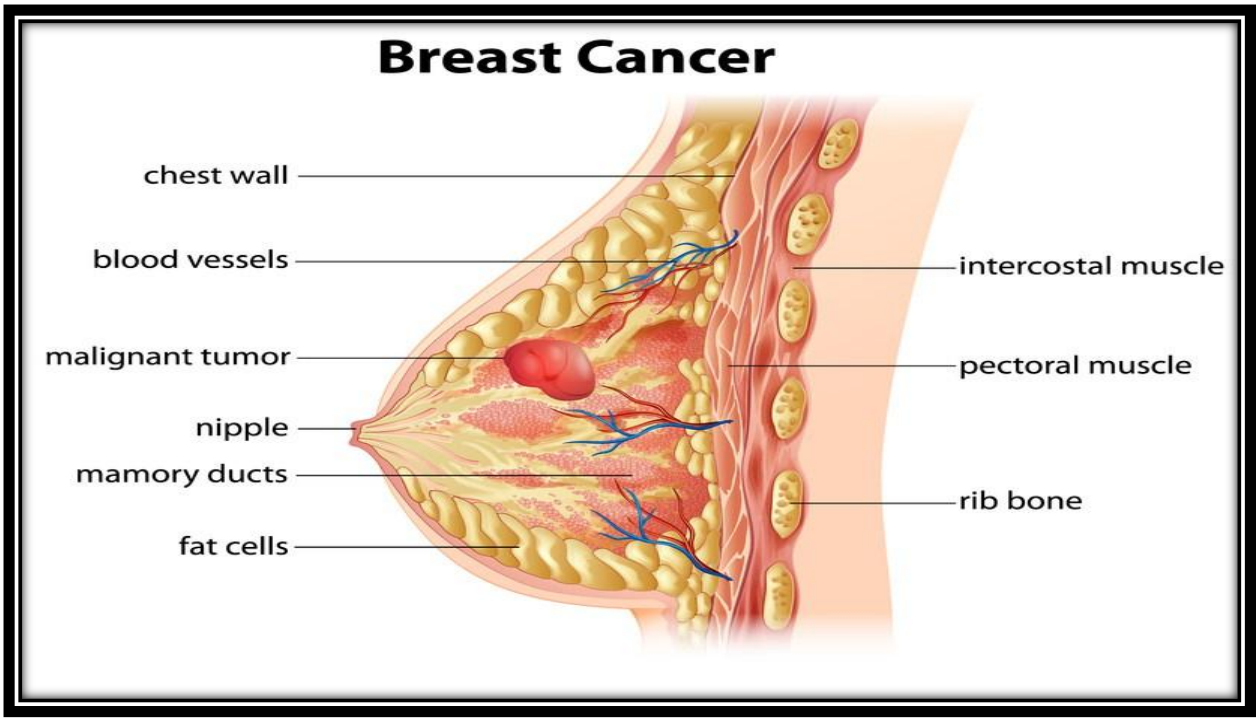

Figure (1-6) Invasive lobular carcinoma (ILC) (Rakha *et al*., 2013)

1.5. Staging of breast cancer.

Table (1-1): Breast cancer stages. (Singletory and Connelly, 2011).

Stage	TNM	Description
0	Tis N0 M0	In ductal carcinoma in situ (DCIS), abnormal cells are in the lining of a breast duct, nodules are not involved and no metastases
IA	T1 N0 M0	Tumour is no more than 2 cms and has not spread to the lymph nodes.
IB	T0 N1 M0 T1 N1 M0	Tumour is no more than 2 cms and cancer cells are found in lymph nodes.
IIA	T0 N1 M0 T1 N1 M0 T2 N0 M0	Tumour is no more than 2 cms and has spread to underarm lymph nodes. Or, tumour is between 2-5 cms, but the cancer hasn't spread to underarm lymph nodes.
IIB	T2 N1 M0 T3 N0 M0	Tumour is 2-5 cms across and has spread to underarm lymph nodes. Or, the tumour is > 5 cms across, but hasn't spread to underarm lymph nodes.
IIIA	T0 N2 M0 T1 N2 M0 T2 N2 M0 T3 N1 M0 T3 N2 M0	Tumour is < 5 cms across, and has spread to underarm lymph nodes that are attached to each other or nearby tissue. Or may have spread to lymph nodes behind the breastbone. Or it is > 5 cms across and has spread to underarm lymph nodes that may be attached to each other or nearby tissue. Or may have spread to lymph nodes behind the breastbone but not spread to underarm lymph nodes.
IIIB	T4 N0 M0 T4 N1 M0 T4 N2 M0	Tumour can be any size and has grown into the chest wall or the skin of the breast. The breast may be swollen or have lumps. It may have spread to underarm lymph nodes, and these lymph nodes may be attached to each other or nearby tissue. Or it may have spread to lymph nodes behind the breastbone.
IIIC	Any T N3 M0	Tumour can be any size, and it has spread to lymph nodes behind the breastbone and under the arm. Or has spread to lymph nodes above or below the collarbone.
IV	Any T Any N M1	Tumour can be any size and cancer cells have spread to other parts of the body.

T = Status of primary tumour, N = Regional lymph nodes, M = Distant metastases

1.6. Grading of Breast Cancer.

The grading system recommended by various professional organizations internationally World Health Organization (WHO), American Joint Committee on Cancer (AJCC), European Union (EU), and the Royal College of Pathologists (UK RCPath) (Pathology Reporting of Breast Disease, 2005). This system has been validated subsequently in multiple independent studies (Rakha *et al*., 2013). It is based on the evaluation of three morphological features: (a) degree of tubule or gland formation, (b) nuclear pleomorphism, and (c) mitotic

The Nottingham Modification of the Bloom-Richardson System depending on the following parameters (Rosai and Ackerman's, 2004):-

Tubular formation

(1)Point: Tubular formation in > 75% of the tumor

(2) Point: Tubular formation in 10 to 75% of the tumor

(3)Point: Tubular formation in <10% of the tumor

Nuclear pleomorphism

(1)Point: Nuclei with minimal variation in size and shape.

(2)Point: Nuclei with moderate variation in size and shape.

(3)Point: Nuclei with marked variation in size and shape.

Mitotic count

(1)Point: 0-9 mitoses/per 10 fields under x25 objective depending on type of microscope e.g. light microscope

(2)Point: 10-19 mitoses

(3)Point: 20 or more mitoses

These result in a total score of between 3and 9 Points, which is translated into the final grade by the following formula:

3 to 5 Points = Grad I (Well–differentiated)

6 to 7 Points = Grad II (Moderately differentiated)

8 to 9 Points = Grad III (Poorly differentiated).

1.7. Epidemiology of Breast Cancer among Iraqi Individuals

In Iraq, breast cancer is the commonest type of malignancy among the Iraqi population in general. It accounts for more than one third of the registered female cancers according to the latest Iraqi Cancer Registry which shows a trend for the disease to affect younger age groups. The number of new cases reported in 2012 was 2906, with an incidence rate of 9.43/100 000 population in both sexes and 18.45/100 000 in female population (Iraqi Cancer Registry 2014).

Within the last two decades, there has been an obvious increase in the incidence rates of breast cancer, which became one of the major threats to Iraqi female health. Many cases in Iraq tend to be diagnosed at advanced stages with a likely prevalence of more aggressive tumor behavioral forms illustrated pathologically; thus yielding

a high mortality incidence ratio (Alwan NA 2000,). According to Globocan 2010, it has been estimated that breast cancer is responsible for 23% of mortalities from malignancies among Iraqi females. A study conducted in Iraq on 721 women presenting with palpable breast masses demonstrated that 14.3% were diagnosed with cancer. Approximately one third of the breast cancer patients presented at age 40–49 years; 71.9% came from urban areas; and 75% were married. History of lactation was reported in 63.1% and hormonal therapy in 29%. Positive family history was recorded in 16.2%. Although the lump was detected by the patient herself in 90.6% of cases, only 32% sought medical advice within the first month; accordingly classifying 47% of these patients in advanced stages (III and IV). (Alwan NA - 2010)

Another study applied on a sample of educated women affiliated to two prominent universities in Iraq documented that almost 75% of the participants believed that the best way to control breast cancer was through early detection and other possible preventive measures. In spite of the finding that most participants (90.9%) had heard of BSE, however, only 48.3% practiced this maneuver; the most common reason behind not doing so was lack of knowledge of how to perform the technique correctly. (Alwan NA – 2012) The findings of these studies justify increasing efforts for establishing comprehensive breast cancer control programs in Iraq focusing on early detection and promoting public awareness.

1.8. The risk factors for breast cancer.

Different cancers have different risk factors. For example, exposing skin to strong sunlight is a risk factor for skin cancer. Smoking is a risk factor for cancers

of the lung, mouth, larynx, bladder, kidney, and several other organs. Having a risk factor, or even several, does not mean that you will get the disease, since women who have one or more breast cancer risk factors never develop the disease, while many women with breast cancer have no apparent risk factors **(**Aguilar *et al***.,** 2012).

Even when a woman with risk factors develops breast cancer, it is hard to know just how much these factors might have contributed. Some risk factors, like a person's age or race, can't be changed. Others are linked to cancer-causing factors in the environment. Others are related to personal behaviors, such as smoking, drinking, and diet. Some factors influence risk more than others, and your risk for breast cancer can change over time, due to factors such as aging or lifestyle (Hartmann *et al*., 2005).

1.7.1. Gender.

Being a woman is the main risk factor for developing breast cancer. This disease is about 100 times more common among women than men. May be due to the fact men have less of the female hormones estrogen and progesterone, which can promote breast cancer cell growth (Boughey *et al.*, 2006).

1.7.2. Age.

Breast cancer incidence increases with age. Being rare before the age of 25, then incidence rises, increasing steeply with age 30–49. After age 50, breast cancer incidence continues to more increasing with the oldest ages **(**Howlader *et al*., 2013).

1.7.3. Hormones.

For years, estrogen has been a suspected carcinogen based on several epidemiological evidence which associate the estrogen to breast, endometrial, and uterine cancers. Women who begin menstruating early, or who start menopause late, produce more estrogen over their lifetimes and have a higher risk of breast cancer. Recently, the clinical trial of estrogen plus progestin treatment therapy was terminated because of an increased risk of breast cancer (Dahlman *et al*., 2011).

Estrogen was originally believed to cause cancer by stimulating cells proliferation. After the hormone binds to its receptors in a cell, it turns on hormone-responsive genes that promote DNA synthesis and cell proliferation. If a cell already had cancer-causing mutations, those cells will also proliferate and have a chance to grow into tumors. But if cell proliferation occur only via receptor-mediated processes is the only mechanism, then all estrogens should cause cancer (Björnström and sjoberg, 2005).

Estrogen metabolism may play a key role in estrogen-induced cancers because different estrogens differ their intracellular metabolic pathway. The cell uses a series of reactions to rid itself of estrogen. In metabolizing carcinogenic estrogens, the reactions produce intermediates capable of producing oxygen radicals that can damage the cell's fats, proteins, and DNA. Unrepaired DNA damage can turn into a mutation, which can later promote cancer (National Cancer Institute, 2006).

1.9. Breast Cancer Immunity

The idea that the immune system can control cancer has been a subject of debate, but recently it has become generally accepted that the immune system has the ability not only to prevent tumor growth but also to promote it through a process called immunoediting, and this process is comprised of three phases: elimination, equilibrium and escape (Schreiber *et al*., 2011). Elimination is achieved through identification and destruction of nascent transformed cells by acute tumor-inhibiting inflammation, characterized by infiltration of effector cells of the innate and adaptive immune system, as well as production of tumorinhibiting cytokines. The escape phase is sustained by chronic tumor-promoting inflammation, which mainly involves immunosuppressive cells and soluble factors (Vesely *et al*., 2011). Evading immune destruction has recently been recognized as a hallmark of cancer, and in general, the use of immunosuppressants following organ transplantation or HIV infection has been shown to increase the risk of tumors such as skin cancer, non-Hodgkin's lymphoma or lung cancers, but not cancers of organs such as breast, brain, prostate and ovary (Hanahan and Weinberg, 2011). These studies suggest that breast cancer cells may be less immunogenic or may take longer to develop. Historically pre-existing inflammation or infection was not considered to be an underlying risk factor for the development of breast cancer. However, it is now clear that the infiltration of leukocytes can either eliminate or promote the development of breast cancers (Coussens and Pollard, 2011).

Several studied have also shown that immunity and inflammation-associated gene expression signatures are able to predict or classify tamoxifen-resistant breast cancers (Vendrell *et al*., 2008). This supports the notion that endocrine resistance is associated with a dysregulated immune response and/or excessive inflammation in the tumor microenvironment (Osborne and Schiff, 2011). A further recent study

suggests that the immune response profile and inflammatory signature in breast cancer may provide useful information on patient prognosis and treatment (Kristensen *et al*., 2012).

1.10. Toll like Receptors (TLRs).

The transmembrane Toll protein was first discovered in the fruit fly Drosophila melanogaster and was originally known only for its function in embryogenesis (Hansson and Edfeldt, 2010).

Evidence then demonstrated its role in defense against fungal and bacterial infections in the fruit fly. A similar role in the mouse was also discovered where defects in the bacterial cell wall component lipopolysaccharide (LPS), mediated immune responses were caused by mutations in the TLR4 gene (Delneste *et al*., 2007). Then, TLRs have been described in insects and vertebrates including humans and the chicken .Structurally similar proteins which include the functionally crucial toll/interleukin-1 receptor (TIR) domain and leucine-rich repeats (LRR) (Figure 1-7) have also been reported in plants ,emphasizing their broad conservation throughout evolution and thus their fundamental importance in defense against pathogens (Jordan *et al.,* 2008).

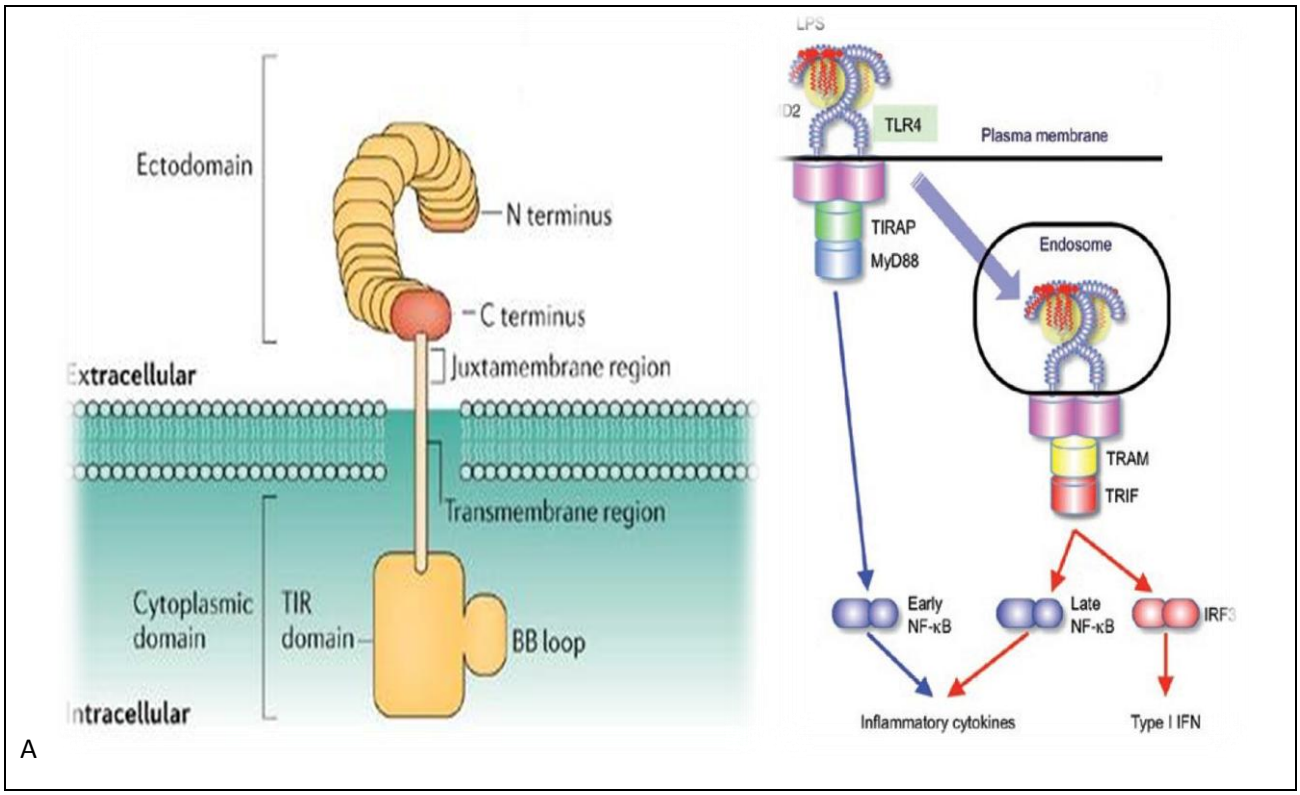

Figure (1-7) TLR structure and signaling (Jordan *et al.*, 2008)

Basic TLR structure and signaling. (A) The divergent ligand binding ectodomain of all TLRs consists of multiple leucne rich repeats (LRRs) interspersed with cysteine-rich regions. There is a short transmembrane domain linking the ectodomain to the highly conserved intracellular TIR domain which bears close resemblance to the IL-1R intracellular domain. (B) Recognition of a conserved pathogen associated molecular pattern (PAMP) by TLR4 causes TLR activation, typically by dimerization and subsequent TIR-TIR domain interactions and autophosphorylation. This induces a downstream signalling process involving a MyD88 dependent or MyD88 independent pathway which culminates in the transcription of inflammatory and anti-viral cytokines (Kawai *et al.*, 2010).

TLRs function by recognizing conserved structural motifs, or pathogen associated molecular patterns (PAMPs) which are inherent to infectious organisms

and rarely found in the host. These are unique to pathogens and allow the host immune system to distinguish non-self from self and thus initiate an intracellular signaling cascade which can bring about the appropriate innate and adaptive immune response. There are 10 functional human TLRs, each with their own particular ligand(s) specificity and effector function. For example, TLR2 can be heterodimerized with either TLR1 or TLR6 to recognize triacylated or diacylated lipopeptides respectively (O'Neill *et al*., 2010).

TLR4 recognizes LPS ingredients of the cell wall of Gram negative bacteria, whereas TLR3 and TLR9 recognized signature dsRNA (dsRNA) and unmethylated -cytosine-phosphate-guanine (CpG) motifs, respectively. TLR5 detects flagellin, a constituent of bacterial flagella and TLR 7 and TLR8 sense single-stranded viral RNA (ssRNA) (Lu and Sun, 2012).

TLR4 has been extensively investigated and demonstrated to be essential for tumor antigen cross presentation in mouse models (Apetoh *et al*., 2007). The gene encoding for TLR4 is located on chromosome 9q32-q33, contains 4 exons and is expressed on lymphocytes, monocytes, macrophages and DC. TLR4 binds microbial ligands such as lipopolysaccharide (LPS) (Kutikhin, 2010). The subcellular localization of TLR4 is unique because it is localized to both the plasma membrane and endosomal vesicles. TLR4 is major TLR and have been actively investigated in inflammation and cancer. There is evidence that TLRs, particularly TLR2 and TLR4, directly regulate major proinflammatory and host defense functions of human neutrophils (Sabroe *et al*., 2005). TLR4 ligation on tumor cells can enhance the secretion of immunosuppressive cytokines and induce resistance to TNF-related apoptosis inducing ligand (TRAIL)-induced apoptosis (Chen *et al*., 2008). Studies have shown that lipopolysaccharide (LPS) ligation to TLR4 promotes tumor cell adhesion and invasion in a murine model by acting NF kappa

B and the silencing of TLR4 increases tumor progression and metastasis in a murine model of breast cancer (Wang *et al.*, 2012).

1.11. Innate Immunity and Toll-like receptors.

The innate immune system involve a group of systemic defenses which have principle role in eliminating an infection or to control it in check until the pathogen can be recognized and cleared by the adaptive immune response. These defenses range from relatively simple yet effective physical barriers such as the body's epithelia or the respiratory tract's cilia to those at the molecular level such as the production of nitric oxide by macrophages to degrade phagocytosed pathogens (O'Neill, 2013). Toll-like receptors (TLRs) are one such class. Belonging to a molecular recognition and signaling system, these type 1 transmembrane glycoproteins represent a link between the innate and adaptive immune responses (Aderem and Ulevitch, 2000)

1.12. TLR adaptor proteins.

The diffusion downstream of TLR signaling involves the recruitment of appropriate protein that bind to the cytoplasmic TIR-domain of TLRs through their own intrinsic TIR-domain adapter proteins. There are four adapter proteins that activate TLR-myeloid differentiation factor 88 (MyD88), MyD88 adapter-like (MAL); also known as Toll-IL-1 adapter protein (TIRAP) TIR-domain-containing adapter inducing IFN-β (TRIF) and TRIF molecule related adapter (TRAM).These adapters couple downstream protein kinases that ultimately lead to the activation of transcription factors such as nuclear factor-kB (NF-kB) and factor-regulating members (IRF) family of interferon (IFN). The critical domain common to all five

of the TLR adapters and the TLRs themselves is the TIR domain. This is located on the cytoplasmic portion of all TLRs and allows the domain join TIR-reciprocal and TIR-domain on the exposed surface of the adaptor molecule (Kawai *et al.,* 2010).

The IL-1 receptor (IL-1R) also contains a TIR- domain hence the existence of the TLR/IL-1R superfamily. Despite the fact that the TLRs having somewhat similar signal transduction pathways, there is specificity with regard to their adaptor usage. MyD88 is the common downstream adaptor that is recruited by all TLRs, except TLR3. MAL is required for TLR4, and to a lesser extent, TLR2 signaling .TRIF mediates TLR3 and TLR4 signaling. Finally, TRAM mediates TLR4 signaling exclusively, acting as a bridging adaptor to recruit TRIF to the TLR4 complex. In addition, an inhibitory TLR adaptor protein called sterile alpha and TIR motif-containing protein (SARM) has also been identified which negatively regulates TRIF mediated signaling (Yamamoto *et al*., 2013).

TLR4 is the only TLR whose activation utilizes all five TLR adaptor proteins and consequently, its signaling is split into two broad categories according to its use of the MyD88 adaptor. The 'MyD88 dependent' pathway is used by all TLRs except TLR3. TLR3 utilizes TRIF only (Figure 1-8). The 'MyD88 independent' pathway uses the adapters TRAM and TRIF in the case of TLR4 signaling to activate anti-viral and late-stage inflammatory responses. Therefore, the function of the adaptor proteins is to provide specificity to TLR signaling in order to organize the resulting cytokine profile to efficiently defend against the infectious agent. Thus, TLR engagement in response to a PAMP or DAMP encourages the recruitment of the relevant TLR adaptor protein(s) which provides a docking platform for downstream effector signaling molecules. This culminates in the production of proinflammatory cytokines, chemokines, and antimicrobial type I

IFNs (IFN-β and IFN-α) which serve to trigger an inflammatory and/or antimicrobial immune response to limit the infectious agent.

TLRs are expressed on cells of the immune system but there is growing evidence that TLRs are also expressed on tumor cells, where they may influence tumor growth and host immune responses (Iwasaki *et al*., 2004)

Activation of TLR expressed on tumor cells may have profound consequences for tumor growth by release of inhibitory cytokines, inflammatory factors, proteinases, and other small molecules such as nitric oxide (Allavena *et al*., 2008). Recent evidence suggests that TLRs also contribute to tumor-cell resistance to apoptosis and increased invasiveness (Yang *et al*., 2010)

The human breast cancer cell line MDA-MB- was found to express TLR1-TLR10 at both the mRNA and protein levels. TLR4 was found to be the highest expressed among the TLRs in MDA-MB-231. Knockdown of TLR4 gene in MDA-MB-231 resulted in a dramatic reduction of breast cancer cell viability and inhibition of IL-6 and IL-8 cytokines, compared with vector control (Xie *et al.*, 2009). Another study highlights the role of TLR9 in highly invasive MDA-MB- breast cancer cell line which when activated promotes MDA-MB-231 cell invasion by increasing the activity of matrix metalloproteinase 13 (MMP13), but not MMP8 . Samples of mammary carcinomas with recurrence have also exhibited a significant increase in the mRNA levels of TLR3, TLR4 and TLR9 (Bauer *et al.*, .2005). A significant percentage of tumors also showed TLR4 expression by mononuclear inflammatory cells (21.6%) and TLR9 expression by fibroblast-like cells (57.5%). Tumors with high TLR3 expression by tumor cell or with high TLR4 expression by mononuclear inflammatory cells, but not TLR9-high fibroblast like cells were significantly associated with higher probability of metastasis (Yuzheng *et al.*, 2012). Expression of TLR9 isoforms A and B has been detected in clinical breast

cancer specimens. Expression of TLR9 and its invasive effects on breast cancer cells was regulated by ERα and sex steroid hormones. TLR9 expression was also found to be commonly used hormonal cancer therapy bicalutamide (Rajput *et al*., 2013).

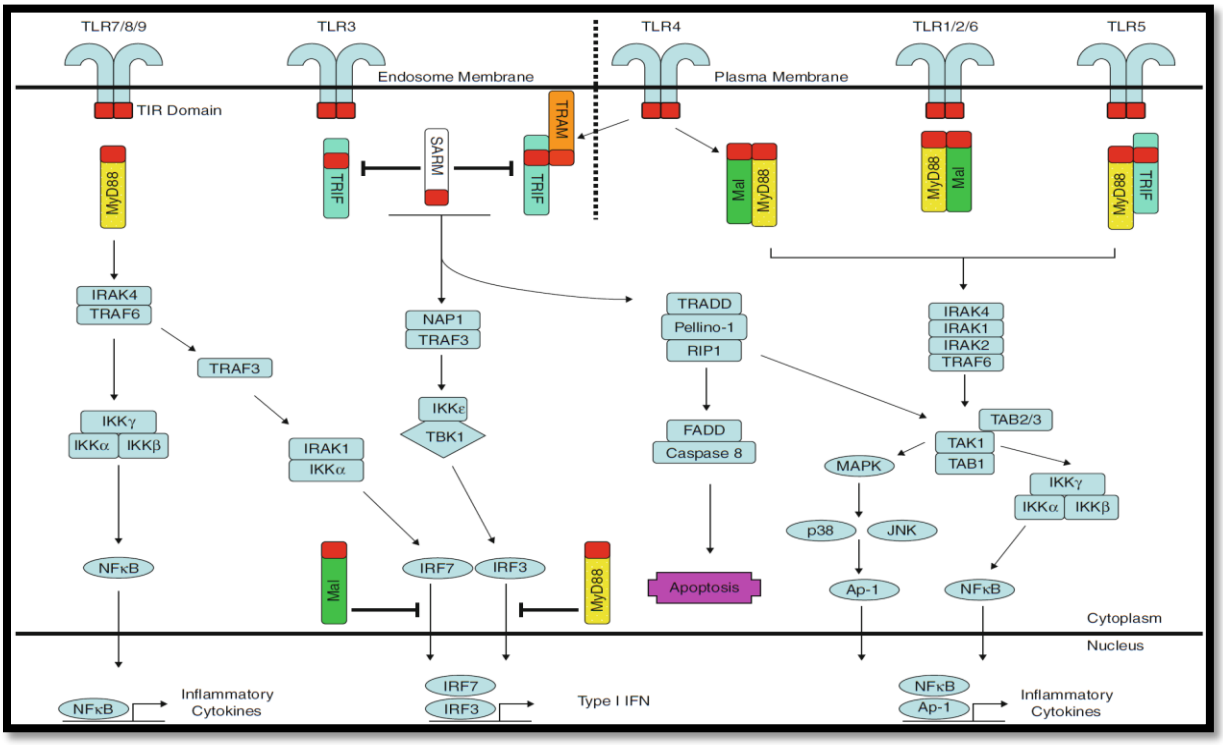

Figure (1-8) Overview of TLR signaling the role of the TIR-domain containing adaptors. (Kawai *et al*., 2010).

1.13. Genetic alterations in breast cancer

Molecular oncology is one of the most promising fields that may contribute considerably to diagnosis of breast cancer and its metastases addressing major problems with early detection, accurate staging, and monitoring of breast cancer patients (Jwad, 2012). Genetic testing for mutations in breast cancer susceptibility genes offers some women and their families the opportunity for risk-reducing intervention (Teo *et al.*, 2013). medical risk reduction gene-targeted therapeutics

(Tutt *et al.*, 2010).Breast cancer development can be triggered by mutations of the signals in the network that controls cell division, and can be associated with genetic predisposition (e.g., mutations in BRCA1 and BRCA2genes), exposure to some environmental factor (e.g., radiation exposure of the chest), or both, So it is an interplay between genetic changes and environmental factors. The predisposition for breast cancer is inherited but not everyone will develop cancer (Al Bederi, 2011).

1.14. MyD88 gene

Human myeloid differentiation factor 88 (MyD88) was first identified in 1990 as the 88 th gene that was induced during the terminal differentiation of myeloid precursor cells in response to IL-6 (Yamamoto *et al.*, 2013). It is 296 amino acids (a.a) in length and contains three domains: an N- terminal death domain (DD) which enables interactions with downstream DD containing proteins, an interdomain, and a C-terminal TIR domain which facilitates homotypic interaction with other TIR-containing proteins (Figure 1.9) (Jenkin and Mansell *et al.*, 2010). MyD88 is 296 amino acids (a.a) in length and contains two domains. At the C terminus (a.a 1–110) is the death domain (DD) and at the N terminus (a.a 155–296) is the TIR domain (watters, *et al.*, 2007).

MyD88 exists primarily in the cytoplasm of resting cells wherein it is belived t to exist in a weak and reversible oligomerised form. Upon TLR activation and consequent TIR-dimerization, MyD88 binds to the TLR complex via its TIR-domain, thus stabilising its oligomeric form to provide a platform for downstream molecules to bind via DD-DD interactions. Interleukin-1 receptor-associated kinase 4 (IRAK4), is the critical downstream molecule of MyD88, being absolutely

required for MyD88 dependent signaling and for the recruitment of further downstream molecules, IRAK1 and IRAK. Complex formation is hierarchical whereby MyD88 first recruits IRAK4. This complex is required to recruit IRAK2 or the related IRAK1. The resulting structure, also called the Myddosome and serves to bring the kinase domains of the IRAK molecules into close proximity to initiate their auto phosphorylation (Lin *et al.*, 2010).

Phosphorylation of the IRAKs recruits the E3 ubiquitin ligase, TNFR-associated factor -6 (TRAF6) which ubquitinates itself. TRAF6 utilizes its E3 ubiquitin ligase activity to ubiquitinate a scaffolding protein, NFκB essential modulator (NEMO) (kang *et al.*, 2011). The combination of TRAF6 mediated ubiquitin chain formation and NEMO functions to recruit TGF-β activated kinase 1 (TAK1) which itself recruits the TAK-1 binding proteins 1 (TAB1) and TAB2. TAK1 phosphorylates IKKβ as well as initiating a MAP kinase cascade leading to activation of the transcription factor CREB. IKKβ phosphorylates the inhibitors of κB (IκB) α and IκBβ leading to NFκB release and nuclear translocation to bind the promoter regions of pro-inflammatory cytokines such as TNFα and IL-12 (Figure1-9).

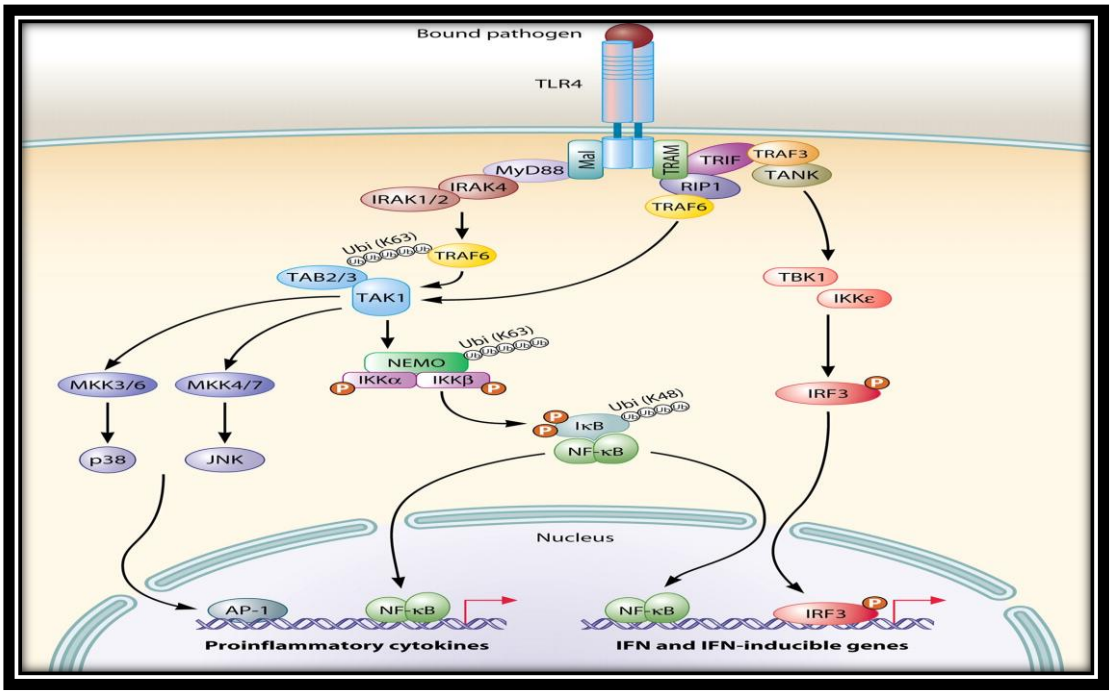

Figure (1-9): MyD88 dependent TLR signaling pathway. (Lin *et al*, 2010).

1.15. Location of the MYD88

Cytogenetic Location: 3p22

Molecular Location on chromosome 3: base pairs 38,138,478 to 38,143,022.

The MYD88 gene is located on the short (p) arm of chromosome 3 at position 22.

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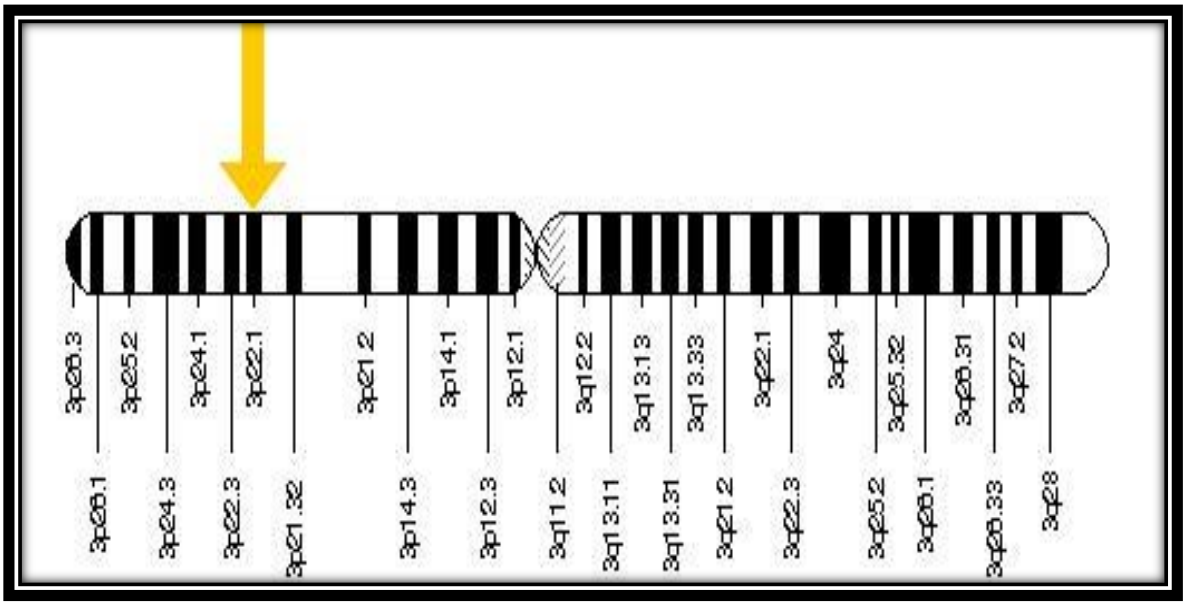

Figure (1-10): Location of the MYD88 (Ngo et al., 2011).

1.16. Immunohistochemistry (IHC)

Used to characterize intracellular proteins (antigens) or various cell surfaces in all tissues in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues (Ramos and Miller, 2014).

Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death (apoptosis). Immunohistochemistry is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue (Bilous *et al*., 2003).

The direct method is a one-step staining method and involves a labeled antibody (e.g. FITC-conjugated antiserum) reacting directly with the antigen in tissue sections. While this technique utilizes only one antibody and therefore is simple and rapid, the sensitivity is lower due to little signal amplification, in contrast to indirect approaches. The indirect method involves an unlabeled primary antibody (first layer) that binds to the target antigen in the tissue and a labeled secondary antibody (second layer) that reacts with the primary antibody. The secondary antibody must be raised against the IgG of the animal species in which the primary antibody has been raised. This method is more sensitive than direct detection strategies because of signal amplification due to the binding of several secondary antibodies to each primary antibody if the secondary antibody is conjugated to the fluorescent or enzyme reporter. Further amplification can be achieved if the secondary antibody is conjugated to several biotin molecules, which can recruit complexes of avidin-, streptavidin- or NeutrAvidin protein-bound enzyme. The difference between these three biotin-binding proteins is their individual binding affinity to endogenous tissue targets leading to nonspecific binding and high background (Barnes *et al*., 1996).Visualizing an antibody-antigen interaction can be accomplished by an antibody is conjugated to an enzyme, such as peroxidase, that can catalyze a color-producing reaction (figure 1-11). Alternatively, the antibody can also be tagged to a fluorophore, such as fluorescein or rhodamine (Coons *et al*., 2000).

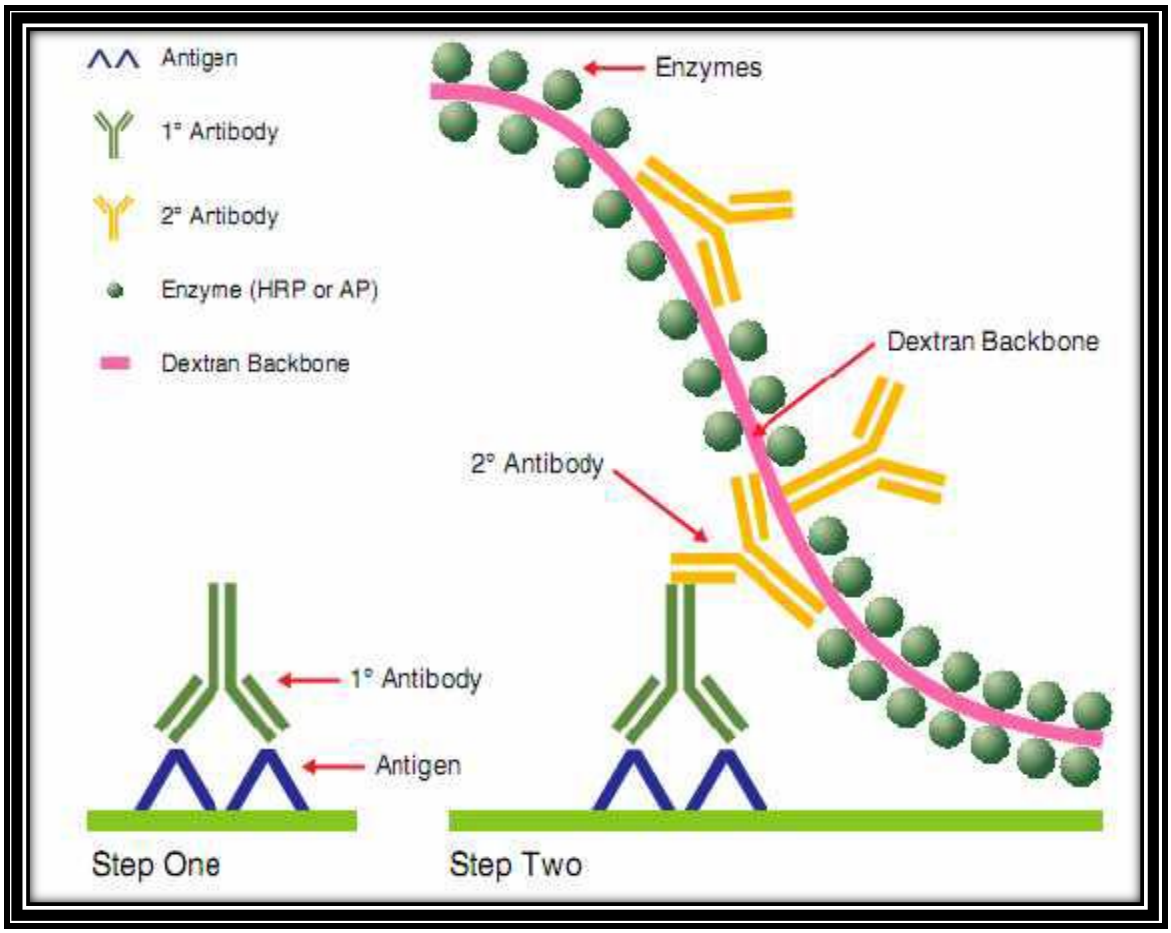

Figure (1-11) Two-step polymer method (Envision method) (Kumar and Rudbeck, 2009).

Ministry of Higher Education
And Scientific Research
Al- Nahrain University
College of science
Department of Biotechnology

Molecular and Immunohistochemical Study on Breast Cancer female Patients in a Sample of Iraqi Population

A Thesis
Submitted to council of College of Science, Al-Nahrain University as a partial fulfillment of the requirements for the degree of Doctorate of Philosophy in Biotechnology

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October 2016 **Moharm 1438**

Supervisors Certification

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In view of the available recommendations, I forward this dissertation for debate by the examining committee.

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Title: Head of Biotechnology Department

Date: / / 2016

Committee Certification

We, the examining committee, certify that we have read this dissertation entitled " Molecular and Immunohistochemical Study on Breast Cancer Patients in a Sample of Iraqi Population" and examined the student "Sara Salih Hassan" in its contents and that, in our opinion, it is accepted for the degree of Doctorate of Philosophy in Biotechnology.

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I hereby certify upon the decision of the examining committee.

Signature:
Name: Dr. Hadi M. A. Abood
Scientific Degree: Professor
Title: Dean of College of Science

Summary

Breast cancer is the most common cancer of women worldwide, Accounting for 25% of all cancers, with an estimated 1.57 million new cases in 2016. n Iraq, According to the latest Iraqi Cancer Registry, breast cancer is the most common malignancy among the Iraqi population THAT constitutes about one fourth of the registered cancer cases and it is the leading cause of death among the Iraqi population. The study aimed to detecting Immunohistochemical and molecular abnormalities in myeloid differentiation primary response gene 88 (**MYD88**) and Toll like Receptor 4 (TLR4) genes in Iraqi patients with breast carcinoma. The present study was conducted at AL- Emamain AL- Kadhemaian teaching Hospitals in Baghdad from April to November 2013 included 50 samples of female patient with malignant breast tumor, twenty samples of patient with benign breast tumor, and 25 blood samples from healthy individuals as controls. DNA extraction was performed from formalin fixed, paraffin embedded (FFPE) sections. After DNA quantification, the DNA samples were used for Conventional polymerase chain reaction (PCR) amplification of Toll like receptor 4 (TLR4) exon (1,2,3,4) by using five sets of primers and Human myeloid differentiation factor 88 (MyD88) gene exon (١,5) by using primer sets .the result revealed that The most of malignant BT patients were observed at the age group ≥ 40 years (٦٦%), while in benign breast tumor patients were observed at the age group ≤ 40 years (60.0%). There is significant difference at malignant BT patients (P ≤ 0.01). agarose gel electrophoresis of the polymerase chain reaction (PCR) products of Toll like respecter 4 (TLR4) showed the presence of bands with size of 853 bp, 967 bp, 919 bp, 720 bp, polymerase chain reaction (PCR) products of myeloid differentiation primary response gene 88 gene showed the presence of bands with size of 606 bp, 877 bp. PCR products were purified, and their DNA sequencing were performed,

and analyzed by National Center for Biotechnology Information (NCBI). the result revealed that TLR4 have 21 mutations which are 12(57.14%) substitution, 7 (33.34%) deletion and 2 (9.52%) insertion. there is a significant difference among malignant BT patients groups ($P<0.01$). In myeloid differentiation primary response gene 88 gene the result revealed that substitution 3(100%). No insertion and no deletion in this gene. The differences in this study were significant ($P<0.01$) among malignant BT patients groups. Immunostaining was used to determine the positive expression of Toll like receptor 4 (TLR4). Among 30 samples of malignant breast tumor patient, 5(16.67%) have grad I, 13(43.33%) have grad II, and 12(40%) have grad III. difference ($p<0.01$) in these values in malignant BT patient. Immunohistochemical analysis of Toll like receptor 4 (TLR4) that conducted on 30 samples of malignant BT patient cleared that 24 (80%) patient had positive expression for Toll like receptor 4 (score +2&+3), while 6 out 30 samples (20%) scored +1, which is considered Toll like respecter 4 (TLR4) negative expression. Furthermore, 6 out of 15 (3.33%) samples from benign breast tumor scored +1, and 9 out of 15(60%) scored +2&+3.

أ لا هـد ا ء

الى من كلل العرق جبينه... وشققت الأيام يديه... الى من علمني أن ألاعمال الكبيره لا تتم ألا بالصبر والعزيمة وألاصرار... الى والدي أطال الله بقاءه ...وألبسه ثوب الصحة والعافية.. ومتعني ببره ورد جميله أُهدي ثمره من ثمار غرسة .....والدي

ألى من نذرت عمرها في أداء رسالة صنعتها من أوراق الصبر وطرزتها في ظلام الدهر على سراج ألامل بلا قيود او كلل رسالة تُعلم العطاء كيف يكون العطاء وتُعلم الوفاء كيف يكون الوفاء اليك أمي اهدي هذه الرسالة وشتان بين رسالة ورساله الى روح حبيبتي أهدي روحي وكل طموحي .....أمي رحمها الله

بكل حب... الى رفيق دربي... الى من سار معي نحو الحلم خطوه بخطوه ...بذرناه معا... وحصدناه معا وسنبقى معا بأذن الله .....الى زوجي جزاك الله خير جزاء

الى تؤام روحي... الى من أرى التفائل بعينيها والسعادة في ضحكتها... الى التي بمحبتها أزهرت ايامي وتفتحت براعم الغد ... الى صاحبة القلب الطيب والنوايا الصادقة... الى من سرت معها الدرب خطوة بخطوة وماتزال ترافقني حتى الان ....همسه

الى من هن اقرب الي من روحي... الى من شاركنني حضن الام و منهن استمد عزتي واصراري ....الى اخواتي

اهدي أليكم جهدي المتواضع

سـا ر ه

﴿بِسْمِ اللهِ الرَّحْمنِ الرَّحِيمِ﴾

{يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ}

(المجادلة: ١١)

الخلاصة

سرطان الثدي هو السرطان الأكثر شيوعا من النساء في جميع أنحاء العالم وهو ما يمثل ٢٥٪ من جميع حالات السرطان ما يقدر ب ١٥٧٠٠٠٠ حالة جديدة في عام ٢٠١٦.. في العراق، سرطان الثدي يشكل حوالي ربع الحالات المسجلة للسرطان ويعتبر السبب الرئيسي للوفاة بين سكان العراق. وهدفت الدراسة إلى الكشف عن تشوهات كيميائية مناعية نسيجية و جزيئية في جينات MYD88 وTLR4 في المرضى العراقيين الذين يعانون من سرطان الثدي . وقد أجريت هذه الدراسة في المستشفيات التعليمية لمستشفى الاماميين الكاظميين في بغداد من نيسان الى تشرين الثاني ٢٠١٣ وشملت الدراسة على ٥٠ عينة من مريضات مع ورم الثدي الخبيث، وعشرين عينة من مريضات مع ورم الثدي الحميد، و ٢٥ عينة دم من الاشخاص الاصحاء كمجموعة سيطرة. تم إجراء استخراج الحمض النووي من عينات من انسجة مثبتة بشمع البارافيين مغمورة في بالفورمالين. بعد التقدير الكمي للحمض النووي، استخدمت عينات من الحمض النووي في تفاعل البلمرة المتسلسل (PCR) لتضخيم (TLR4) اكسون (١،٢،٣،٤) باستخدام خمس مجموعات من البادئات و جين MYD88 بأستخدام مجموعة من البادئات. وقد لوحظ ان معظم مرضى ورم الثدي الخبيث في الفئة العمرية ≤ ٤٠ سنة (٦٦٪)، في حين ان مرضى ورم الثدي الحميد في الفئة العمرية ≤ ٤٠ سنة (٦٠.٠٪). هناك فرق معنوي في مرضى ورم الثدي الخبيث ($P \leq 0.01$). أظهرت نتائج الترحيل الكهربائي لهلام الاغاروس لتفاعل البلمرة المتسلسل لجين TLR4حجوم النواتج عند ٨٣٥ زوج قاعدي, ٩٦٧ زوج قاعدي, ٩١٩ زوج قاعدي, ٧٢٠ زوج قاعدي, وأظهرت نواتج تفاعل البلمرة المتسلسل (PCR) لجين MYD88 وجود حزم مع حجم ٦٠٦ زوج قاعدي، ٨٧٧ زوج قاعدي. تم تنقية نواتج PCR، وأجري تسلسل الحمض النووي، وتحليلها من قبل المركز الوطني لمعلومات التكنولوجيا الحيوية (NCBI).

كشفت النتائج عن وجود ٢١ طفرة في جين TLR4 وتشمل (٥٧.١٤٪) الاستبدال، ٧ (٣٣.٣٤٪) الحذف و٢ (٩.٥٢٪) حشر. هناك فرق معنوي بين مجوعة في مرضى ورم الثدي الخبيث ($P <0.01$). في الجين MYD88 كشفت النتائج عن وجود ٣ (١٠٠٪) طفرات استبدال . كانت الفروق المعنوية في هذه الدراسة ($P <0.01$). تم استخدام التصبيغ المناعي لتحديد التعبير الإيجابي لمستقبل (TLR4). ومن بين ٣٠ عينة من مريضات السرطان الثدي (16.67%)5كانوا في المستوى I و (43.33%)13 كانوا في المستوى II و (40%)12 كانوا في المستوى III .. التحليل النسيجي المناعى الكيميائي لمستقبل (TLR4) الذي أجريت على

٣٠ عينة من مريضات ورم الثدي الخبيث أوضحت أن ٢٤ (٨٠٪) من المريضات كان التعبير الإيجابي لمستقبل TLR4 (درجة + ٢ & + ٣)، بينما سجل ٦ من أصل ٣٠ عينة (٢٠٪) +١، الذي يعتبر التعبير السلبي. وعلاوة على ذلك، و ٦ من أصل ١٥ (٣٣.٣٪) عينة من عينات ورم الثدي الحميد سجل +١، و ٩ من أصل ١٥ (٦٠٪) وسجل + ٢ & + ٣.

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جمهورية العراق
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دراسة جزيئية وكيميائية نسيجية مناعية لمريضات سرطان الثدي لعينات من المجتمع العراقي

رسالة

مقدمة الى كلية العلوم- جامعة النهرين وهي جزء من متطلبات نيل

درجة دكتورا فلسفة علوم / التقانة الاحيائية

من قبل

سـا ر ه صـا لـح حسن

ماجستير تقانة احيائية (٢٠١٠)

بإشراف

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تشرين الاول ٢٠١٦

محرم ١٤٣٨