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Immunological and molecular study for TLR2 in patients with breast tumors

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ABSTRACT

A tumor is a mass of abnormal tissue. There are two types of breast cancer tumors: those that are non-cancerous, or 'benign', and those that are cancerous, which are 'malignant' Seventy (70) biopsy of breast tumors patient's women without chemotherapy for DNA extraction for genetic polymorphism of TLR2 and local immunity study for TLR2, fifty (50) blood sample from healthy women as control group. One hundred (100) blood sample from women with breast tumor for polymorphism of TLR2 and evaluated immune markers (TLR2).The age of women in this study ranging from (14-66) years in AL-Hilla-Teaching hospital , Imam Sadiq Hospital and Marjan hospital a period from September 2021 to October 2022.the result appeared that By Enzyme Linked Immunosorbent Assay technique showed that the concentration of TLR2 in serum patients were (9.953±4.606) significantly increased compare with control (6.774±3.855) respectively, The concentrations of TLR2 in serum patients were (5.599±3.550) significantly different compare with concentrations tissue (21.000 ±14.356)respectively , For TLR2 196 to 174 del genotyping the result was significantly only deletion in (G, C , T) between patients with chemotherapy and control) at (p =0.003* , 0.0007* , 0.04*) respectively , also the result significantly for insertion of (G) between patients with chemotherapy and control) at (p =0.01*) conclusion of this research TLR2 196 to 174 del genotyping the result was significantly only deletion in (G, C , T) between patients with chemotherapy and control) at (p =0.003* , 0.0007* , 0.04*) respectively , also the result significantly for insertion of (G) between patients with chemotherapy and control) at (p =0.01*)

1. Introduction

Breast disorders include a variety of ailments. Most breast illnesses are not malignant. A small number of these lesions required little management since they are clinically unremarkable. On the other hand, certain symptoms, particularly if they continue, may have clinical significance and warrant the attention of the attending physician as well as the patient (Alamri *et al*.,2020). Both myeloid (monocytes, macrophages, and dendritic cells) and lymphoid (T lymphocytes and B lymphocytes) immune cell lineages are present in normal breast tissue. Rather than the stroma and fat, immune cells in normal breast tissue are mostly found in the lobules (Degnim *et al*.,2014). Typically, genetically stable cancer-initiating mutations that can predict medication treatment response or resistance are what cause cancer-intrinsic inflammation (Todoric *et al*.,2016).At several phases of the development of breast tumors, there is inflammation linked to cancer, including extrinsic and intrinsic inflammation (Lim *et al*.,2018 ; Comen *et al*.,2018).

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Human epithelium and immunological cells are the main sources of expression for toll-like receptors (TLRs), which are well-conserved pattern-recognition receptors (Mifsud et al.,2014 ; Brubaker et al.,2015) TLRs primarily operate to stimulate the production and release of chemokines and inflammatory cytokines, which in turn initiates the inflammatory response (Palm et al.,2015 ; Johnston and Corr , 2016) It's interesting to note that TLR2, which is expressed on the surface of breast cancer cells, can be stimulated to increase invasive potential by activating nuclear factor kappa B (NFκB) signaling. This makes TLR2 a viable target for therapy in cases of highly invasive breast cancer. Through NF-κB signaling, TLR2 activation on the surface of breast cancer cells has been shown to increase the disease's invasive potential (Xie *et al.*,2010).

Moreover, recent data indicates that TLR2 signaling can help tumor cells evade immune surveillance and the host immune system (Huang *et al*.,2008). TLR2 plays a role in immunomodulation in BCa (Chow *et al*.,2014), and TLR2 polymorphisms are linked to BCa risk (Zhu *et al*.,2013). As a proto-oncogene, TLR2 is highly expressed in the majority of malignancies and is intimately linked to tumor metastasis (Wang *et al*.,2019).

Though the therapeutic relevance of TLR2 in BCa is yet unknown, research (Lu *et al*.,2011) shown that TLR2 agonists improve the effectiveness of HER2-targeted monoclonal antibody treatment. Research on the genetics of the TLR2 gene has shown a polymorphism that results in a deletion of 22 base pairs (−196 to −174 del) in the promoter of the gene. This modification might drastically change the promoter's activity, which would probably result in less TLR2 being transcribed (Noguchi *et al*.,2004).The aim of this research to study the immunological and genetic relationships for TLR2 between patients with breast tumors and healthy (control).

2. Methodology

One hundred sample (100) from woman with breast tumors (70 biopsies and 100 blood samples) ,30 blood from patient with breast cancer after taken chemotherapy) and 50 control apparently healthy involved (blood) from healthy women ,involved 1- Biopsies for local immunity , About five centimeters away from the tumor, outside the marginal zone, is where the tissue was taken. Fresh tissue was promptly placed in a sterile urine cup or plane tube following excision.It has a standard saline solution in it. DNA extraction from biopsies and TLR2 polymorphism 3- Biopsies for measure immune markers mucosal (TLR2).

4-Serum separated from blood sample by using gel tube were collected from patients women who were suffering from breast tumors and healthy women to measure immune marker systemic (TLR2) with age ranging from (14-66) years in AL-Hilla-Teaching hospital , Imam Sadiq Hospital and Marjan hospital a period from September 2021 to October 2022.

2.1 TLR2 196 to 174 del genotype

Asp299Gly The primers for TLR2

The forward 5 -CACGGAGGCAGCGAGAAA-3 and the reverse 5 -CTGGGCCGTGCAAAGAAG-3 genes were amplified. After five minutes of 95 C denature, the DNA underwent thirty seconds of 95 C, forty seconds of 60 C, and forty seconds of 72 C cycles. The last extension phase

was extended to seven minutes. The 3.5% agarose gel electrophoresis and ethidium bromide staining were used to visualize the PCR results.

3. Statistical analysis

SPSS software (version 23 SPSS) was used for the statistical presentation and analysis of the current study. Statistical significance was defined as p 0.05.

3.1 Ethical approval:

The study was conducted in accordance with the Helsinki Declaration's ethical guidelines. The patient's verbal and written agreement was obtained before any samples were taken. The study protocol, subject information, and consent form were reviewed by a local ethics committee, which authorized them in accordance with document M220106 (which has the reference and date of 17/1/2022).17/1/2022) to get this suggestion.

Table (1) g Demographic of subject

Sample collected from100 patients (70 biopsy and 100 blood sample from women before chemotherapy and 30 after chemotherapy)			Control (50 women)	Chi - square	P – value
		Number (percentage %)			
Age years	(14-29)	20 (22%)	18(36%)	7.089	0.029*
	(30-45)	45(50%)	27(54%)		
	(46-66)	25(28%)	5(10%)		
Family history	Present	28(28%)			
	Absent	72(72%)			
Status	Married	67(67%)			
	Unmarried	33(33%)			
Types of breast tumors	Benign breast tumors	58(58%)			
	Malignant breast tumor	42(42%)			

Result and discussion

The patient groups with breast tumors included (100 sample divided into 30 women receiving chemotherapy treatment, and 100 women receiving blood and 70 women receiving biopsy without treatment).The age ranges for the groups between (14-66) were 14-29, 30-45, and 46-66 years old, with 20 (22%), 45 (50%), and 25 (28%), in that order. The study revealed that the second group (30-45) had the most common breast cancers. The study population comprised 72(72%) women who had a family history of breast cancer, and 28(28%) who did not. The percentages and numbers of married and single groups are 67(67%) and 33(33%), respectively, in Table (1).

The results of the study by (Alwan, 2014) in Iraq contradicted the findings of this one, showing that the peak frequency of breast cancer increased with age until menopause, at which point it began to fall. Over half (54.2%) of the patients were in their primenopausal years; 31.9% of the patients were diagnosed between the ages of 40 and 49, and 22.2% were under 40. Additionally, this study contradicted a study (Majid *et al.,*2017) conducted in Sulaymaniyah which found that, throughout Iraq

during the 2001–2012 period, the rate of breast cancer increased significantly between 2006 and 2012 among women aged 60 and above (P <0.001), but not in younger age groups. Additionally suggests that in Sulaymaniyah, the age group of 60 years and older may have had a rise in breast cancer rates (P = 0.047).

The study of (Wojda *et al*.,2006 ; Orta and Günebakan, 2012) found that age was a risk factor for the evolution of breast cancer. They suggested that the rising incidence of cancer in women over 40 years of age could be caused by increased chromosomal damages as a result of repeated divisions in age increasing, which led to the accumulation of mutations in the DNA that cause cancer development. They also found that "the age-related increase in chromosomal harm occurred hurry in women than in men" because the main cause of aging in women was an increase in the level of aberrations and rise in the level of X chromosome damage.

The demographic graphs for Belgium and Rwanda show that, despite comparable total population sizes, Belgium has a similar absolute number of women in the 30- to 49- or 50- to 69-year-old age groups, whereas Rwanda has 2.4 times more women in this age group than in the 50- to 69-year-old age group. When comparing with European countries, the demographic effect accounts for over half of the difference, with 52.2% of malignancies in the 30-49 range and 37.7% in the 50-69 range. Furthermore, mammography screening programs aimed at women aged 50 to 69 are thought to cause an over-diagnosis rate of 12–20%g, which raises the number of diagnoses in that age group in Europe (Katalinic *et al*.,2019).

This study was agreed with the of study (Al-Rawi , 2013) in Erbil Iraqi that showed the woman between 36-49 years old appeared the highest incident of breast cancer (61.1%) mostly of ductal type (75%) of moderately to poorly differentiated grade.

This study revealed that benign breast tumors were more common than malignant ones, which is consistent with studies conducted in Iraq (Nada and Alwan , 2010) and (Hatim *et al*.,2017) that found 210 cases (80%) of benign breast lesions and higher numbers and percentages of benign breast tumors than malignant ones. A study conducted in North Maharashtra showed 78.52% cases of benign breast lesions, whereas a study conducted in South Maharashtra identified 71.15% of cases and 70% of cases of benign breast lesions (Kumar *et al*.,2010) According to a study conducted in east Maharashtra(Rasheed *et al*.,2014), there are 70% of benign breast lesions in east India and 77.7% in north India. Compared to malignant lesions, the incidence of benign breast disease rises in the second decade and peaks in the fourth or fifth decade (Sangma *et al*.,2013).

The study by (Al-Rawi ,2013) which revealed that 36 cases of malignant breast lesions were discovered in the individuals under investigation conflicted with this study. Ages 36 to 49 accounted for the majority of patients (61.1%) with malignant breast lesions. However, it was discovered that 30.6% of individuals who were over 50 had malignant breast lesions. However, women under the age of 35 were found to have 8.3% of malignant breast lesions. In contrast to the study (Abdulsamad *et al*.,2021) conducted in the province of Basra city center, the family history in this study indicated

that 28 (28%) had a lower history of sickness than 72 (72%).

According to the current study, the likelihood of having no family history was higher than that of having one. This study contradicted the findings of the studies (Hsieh *et al*.,1994) (Azzollini *et al*.,2020) (Johansson *et al*.,2021), which demonstrated Numerous international research have demonstrated a favorable correlation between breast cancer and family history, which may vary depending on the age of the afflicted relative.

According to the current study, there are more married patients with breast cancer than single female patients. This finding is consistent with a study conducted in Iraqi Kurdistan (Ali Ghalib *et al*.,2019) that found a statistically significant correlation between marriage and breast cancer, with 86.4% of cases being married compared to 91.4% of controls (P = 0.037).

4. Immunological study

4.1 TLR2 cytokines detection.

Toll-like receptors (TLRs) are widely expressed on tumor cells and are involved in the initiation and progression of breast cancer (Bhattacharya and Yusuf , 2012). Research has demonstrated that activation of TLR2 on the surface of breast cancer cells enhances their capacity to invade by inducing NF-κB signaling (Al-Harras *et al*.,2016). Because aberrant inflammatory responses negatively affect the host, the TLR system needs to be tightly regulated in both the physiological and pathological states (Houssen *et al*.,2016). TLR2 signaling has been implicated in the development of breast cancer in numerous studies, and both receptors have been connected to the activation of other transcription factors, such as NF κB (Yusuf,2014). When comparing the TLR2 concentration in patient serum (9.953) to the control group's mean of 6.774, Table 2 showed a substantial rise. (El-Kharashy *et al*.,2021) supported this study by showing that, in comparison to the control group (1,106.8± 99.93 p/ml; P=0.0001), patients with metastatic (5,997.4±8,585.23 pg/ml) and non-metastatic (2,258.2±1,832.44 p/ml) breast cancer had significantly higher serum sTLR2 levels.

The mean blood level of TLR2 was found to differ considerably between breast cancer patients and healthy individuals Bastara research (Abdulabbas and Shani,2020) with the former having a level 2.117± 1.026 ng/ml and the latter having a level of 0.195±0.044 ng/ml. A 2013 study discovered that serum levels of TLR2 were higher in breast cancer patients than in healthy controls (Al-Ammiri and Al-Derzi,2013). By initiating a signaling cascade that stimulates transcriptional factor NF-κB, which is implicated in the invasion and metastasis of breast cancer, TLR2 expression on breast cancer cells has been connected to the progression of cancer (Huber *et al*.,2004).

Table (2) Concentration of serum TLR2 between patients and control

Parameters	M±SD concentration pg/ml		P- value
	Patients	Control	
TLR2	9.953±4.606	6.774±3.855	0.04*

*(p≤ 0.05) is considered significant

Table (3) displays the results, which indicate that there was no significant decrease in TLR2 concentration between malignant and benign tissue (p = 0.182 and 0.258, respectively). (39) contested the study's findings, demonstrating that the median serum levels of Toll-like receptor-2 in breast cancer patients (malignant tumors) and the case control group (benign tumors) did not differ statistically significantly. Therefore, the study's focus on Toll-like receptor-2 was on breast tumor cases as a whole.

Table (3) Concentration of tissue TLR2 in Malignant and Benign Breast patients

Parameters	Concentration M±SD pg/ ml		P value
	Malignant tissue	Benign tissue	
TLR2	0.258 ± 0.205	0.182 ± 0.0936	0.1

Within Table (4) This study found that the concentration of TLR2 was higher in the patient group (11.826 and 8.967) compared to the control group (6.773). These results were in line with those of (Al-Ammiri and Al-Derzi, 2013), which showed that blood TLR-2 levels were considerably greater in patients cancers.

The idea that this marker contributes to the disease's pathophysiology may be strengthened by this finding. Furthermore, serum TLR-2 produced results with the maximum accuracy and sensitivity when cutoff values were at or above 0.14 ng/ml. Consequently, TLR-2 could be a useful new diagnostic tool, particularly in the early phases and for high-risk individuals.

Table (4) Concentration of TLR2 in serum patients groups and healthy

Parameters	Concentration pg/ml Mg ±SD			P value
	Malignant tumor	Benign tumor	Healthy	
TLR2	11.826 ± 4.305 b	8.967 ± 4.556 Ab	6.773 ± 3.855 a	0.03*

*similar letters in the same column indicate that there is no significant difference ((p< 0.05) .

5. Molecular study

A- The PCR product in patients without chemotherapy

The PCR product of TLR2 196 to 174 del gene was amplified by using specific primer . the PCR product (band) of TLR2 196 to 174 del gene was 286 –bp in patients Figure (1)

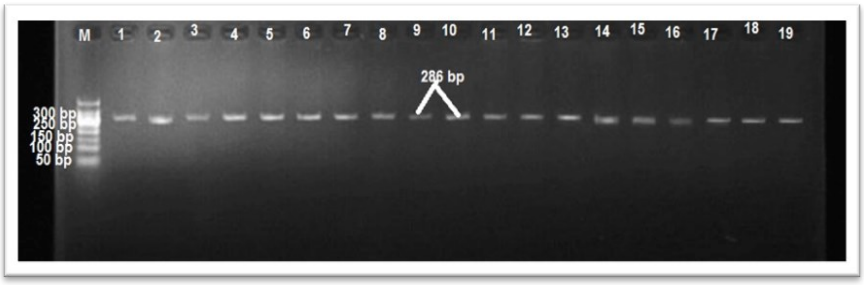

Figure (1) Electrophoreses pattern of PCR product of TLR2 196 to 174 del patients and control blood , M : molecular DNA ladder , 1-19 PCR product . Electrophorases condition : agarose 1.5% , volt 85 , for 1 hour , red safe stain

B- The PCR product in patients in control groups

PCR product of TLR2 196 to 174 del gene was amplified by using specific primer . the PCR product (band) TLR2 196 to 174 del gene was 286 –bp in control patients Figure (2) .

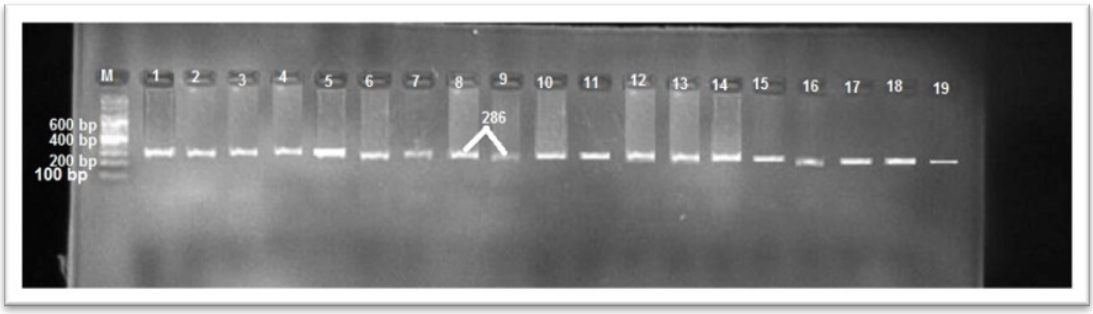

Figure (2) Electrophoreses pattern of PCR product of TLR2 196 to 174 del in blood control , M : molecular DNA ladder , 1-17 PCR product . Electrophorases condition : agarose 1.5% , volt 85 , for 1 hour , red safe stain

DNA sequencing of TLR2 196 to 174 del

To prove the result of TLR2 196 to 174 del, sequencing were ,the result deletion , insertion occur in Guanine , cytosine , thymine and adenine Figure (3).

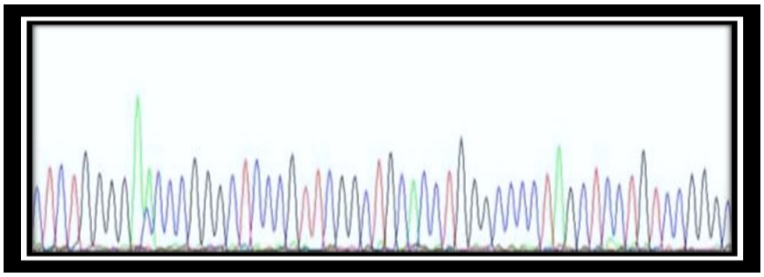

Figure (3) DNA sequencing of TLR2 196 to 174 del

Table (5) Number and percentage for nucleotide deletion between patients serum and Healthy (control)

Nucleotide	Deletion Number / percentage		Odd ratio	P value
	Patients' serum	Healthy (control)		
G	42(49%)	20(43%)	0.758(0.378 – 1.521)	0.4
C	33(39%)	19(41%)	0.688(0.327 – 1.447)	0.3
A	6(7%)	4(9%)	0.705(0.154 – 3.223)	0.6
T	4(5%)	3(7%)	0.345 (0.062 – 1.915)	0.2

In Table (6) appeared the number and percentage of deletion n (G,C,A,T) nucleotide in serum patient were (42(49%), 33(39%) , 6(7%) 4(5%)) , in tissue were(36(45%) , 34(42%) , 8(10%), 2(3%)) respectively.

Table (6) Number and percentage for nucleotide deletion between patients' serum and tissue patients

Nucleotide	Deletion Number / percentage		Odd ratio	P value
	Patients' serum	Patients tissue		
G	42(49%)	36(45%)	0.664(0.396 – 1.195)	0.2
C	33(39%)	34(42%)	0.602(0.319 – 1.136)	0.1
A	6(7%)	8(10%)	0.397(0.104 – 1.503)	0.2
T	4(5%)	2(3%)	0.296 (0.040 – 2.180)	0.2

In Table (7) appeared the number and percentage of deletion in (G,C,A,T) nucleotide in serum patient with chemotherapy were (17(55%) 10(32%) , 3(10%), 1(3%)) , in healthy groups were(20(43%) , 19(41%), 4(9%) , 3(7%)) respectively

Table (7) Number and percentage for nucleotide deletion between patients' with chemotherapy and healthy (control)

Nucleotide	Deletion Number / percentage		Odd ratio	P value
	Chemotherapy	Healthy (control)		
G	17(55%)	20(43%)	0.306(0.139 – 0.674)	0.003**
C	10(32%)	19(41%)	0.208(0.084 – 0.516)	0.0007*
A	3(10%)	4(9%)	0.353(0.063 – 1.964)	0.2
T	1(3%)	3(7%)	0.086 (0.007 – 0.963)	0.04*

In Table (8) appeared the number and percentage of insertion in (G,C,A,T) nucleotide in serum patient were (12(27%) , 11(24%), 6(13%) , 16(36%)) , in healthy groups were(6(34%), 4(22%) , 4(22%), 4(22%)) respectively.

Table (8) Number and percentage for nucleotide Insertion between patients serum and control

Nucleotide	Insertion Number / percentage		Odd ratio	P value
	Patients' serum	Healthy (control)		
G	12(27%)	6(34%)	0.722 (0.245 – 2.121)	0.5
C	11(24%)	4(22%)	1.091 (0.315 – 3.777)	0.8

A	6(13%)	4(22%)	0.705 (0.155 – 3.224)	0.7
T	16(36%)	4(22%)	1.037 (0.262 – 4.103)	0.9

In Table (9) appeared the number and percentage of insertion in (G,C,A,T) nucleotide in serum patient were (12(27%) , 11(24%), 6(13%) , 16(36%)) , in tissue were(5(50%) , 2(20%), 1(10%), 2(20%)) respectively.

Table (9) Number and percentage for nucleotide Insertion between patients and control

Nucleotide	**Insertion**			
	Number / percentage		**Odd ratio**	**P value**
	Patients' serum	**Patients tissue**		
G	12(27%)	5(50%)	1.367 (0.449 – 4.153)	0.6
C	11(24%)	2(20%)	0.948 (0.182 – 4.935)	0.9
A	6(13%)	1(10%)	3.177 (0.329 – 30.623)	0.3
T	16(36%)	2(20%)	1.185 (0.196 – 7.217)	0.8

In Table (10) appeared the number and percentage of insertion in (G,C,A,T) nucleotide in serum patient with chemotherapy were (1(8%) , 4(31%) , 2(15%) , 16(36%)) , in healthy groups were(6(34%), 4(22%), 4(22%), 6(46%)) respectively

Table (10) Number and percentage for nucleotide insertion between patients with chemotherapy and control

Nucleotide	**Insertion**			
	Number / percentage		**Odd ratio**	**P value**
	Chemotherapy	**Healthy (control)**		
G	1(8%)	6(34%)	0.060 (0.006 – 0.524)	**0.01***
C	4(31%)	4(22%)	0.397 (0.091 – 1.721)	**0.2**
A	2(15%)	4(22%)	0.235 (0.035 – 1.564)	**0.1**
T	6(46%)	4(22%)	0.444 (0.100 – 1.974)	**0.3**

6. Conclusion

TLR2 196 to 174 del genotyping the result was significantly only deletion in (G, C , T) between patients with chemotherapy and control) at (p =0.003* , 0.0007* , 0.04*) respectively , also the result significantly for insertion of (G) between patients with chemotherapy and control) at (p =0.01*).

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