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The Association of Five Novel Variants of *TLR7* Gene with Some Biochemical Markers in Breast Cancer Patients from Iraqi Women

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Abstract

Background: The TLR7 gene carries multiple polymorphisms that are likely associated with human diseases, including cancer. The study aimed to shed light on the relationship between the variation of the TLR7 gene and serum level of biochemical markers (CA15-3, CEA, CA125, and CA27-29) in Iraqi women with breast cancer.

Methods: a case-control study involving 100 women volunteers: 50 with breast cancer as a patient group and 50 who appeared to be healthy as a control group. The ages of all participants were ranged between 29 to 75 years. This study was conducted from November 2022 to April 2024 at the Department of Clinical Laboratories, College of Applied Medical Sciences, University of Kerbala . Sanger sequencing was used to investigate variants of the TLR7 gene. The enzyme-linked immunosorbent assay (ELISA) method was used to evaluate the levels of CA15-3, CEA, CA125, and CA27-29 in serum.

Results: Presence of 5 novel unregistered variants in the Intron 2 region. It was found that a significant effect of TLR7 - 12871749 G\C variant (SNP) of the Intron region on serum level of CEA (point biserial correlation coefficient =-0.396, p-value=0.03). A significant effect of TLR7 - 12871764 A\G variant of the Intron region on serum level of CA 15-3 (point biserial correlation coefficient =-0.385, p-value=0.03). The results showed no significant, weak, moderate, or strong associations between all other types of variants when tested individually and the four biochemical markers under study.

Conclusion: Among the five new variations, 12871749 G\C and 12871764 A\G may be the most significant variants because there is a statistical association with some biochemical markers, therefore, Future research should delve deeper into the role of TLR7 polymorphisms in the fields of tumor immunology, which may open new perspective in early diagnosis and prevention of cancer.

ارتباط خمسة متغيرات جديدة في جين *TLR7* مع بعض المعلمات الكيموحيوية عند مرضى سرطان الثدي من النساء العراقيات

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الخلاصة

المقدمة: يحتوي جين TLR7 أشكالًا متعددة من المحتمل أن تكون مرتبطة بالأمراض التي تصيب الإنسان مثل السرطان. هدفت الدراسة إلى إلقاء الضوء على العلاقة بين تباين جين TLR7 و مستوى بعض المؤشرات الكيموحيوية (CA15-3، CEA، CA125، وCA27-29) لدى مرضى سرطان الثدي من النساء العراقيات.

المواد وطرق العمل: دراسة الحالات والشواهد التي تشمل 100 امرأة متطوعة: 50 امرأة مصابة بسرطان الثدي كمجموعة مرضى و50 امرأة تبدو بصحة جيدة كمجموعة ضابطة، تتراوح أعمار جميع المشاركين بين 29 الى 75 عاماً. أجريت الدراسة في الفترة من تشرين الثاني 2022 إلى نيسان 2024 في قسم المختبرات السريرية، كلية العلوم الطبية التطبيقية، جامعة كربلاء. تم التحقيق في تباين جين TLR7 بواسطة طريقة التسلسل Sanger. تم قياس مستويات المعلمات الكيموحيوية في مصل الدم بواسطة طريقة (ELISA).

النتائج: حددت نتائج الدراسة الحالية وجود 5 متغيرات جديدة غير مسجلة في منطقة Intron 2 لجين TLR7. من بين هذه المتغيرات كان هناك فروق ذات دلالة احصائية ($P\leq0.05$) بين (TLR7 - 12871749 G\C SNP) ومستوى CEA في المصل , وبين (TLR7 - 12871764 A\ SNP) ومستوى CA 15-3 , ولم تكن هناك ارتباطات احصائية ضعيفة أو متوسطة أو قوية بين جميع أنواع المتغيرات الأخرى عند اختبارها بشكل فردي مع المعلمات الكيموحيوية الأربعة قيد الدراسة.

الاستنتاج: قد يكون المتغيران 12871749 G\C و 12871764 A\G من اهم المتغيرات لوجود ارتباط احصائي مع بعض المعلمات الكيموحيوية , لذلك ينبغي أن تتعمق الأبحاث المستقبلية في دور تعدد أشكال TLR7 في مجالات علم المناعة المتعلق بالأورام ، والتي قد تفتح آفاقا جديدة في التشخيص المبكر والوقاية من السرطان.

1. Introduction

Toll-like receptors (TLRs) are a type of pattern recognition receptor (PRR), proteins that enhance immunity by identifying damage-associated molecular patterns (DAMP) and pathogen-associated molecular patterns (PAMP) (Amarante-Mendes et al., 2018). TLR agonists, including TLR7, stimulate cytokine responses that promote antitumor immunity by enhancing CD8þ T-cell activation, increasing myeloid-derived suppressor cells (MDSC) maturation, and T-regulatory cells (Treg) inhibition (Tang et al., 2013; Spinetti et al., 2016). In the human body, there are ten TLR members, some of which are located on the cell membrane (TLR1, TLR2, TLR4, TLR5, and TLR6), while others in endosomes (TLR3, TLR7, TLR8, and TLR9) (Chi et al., 2017). One member of the TLRs family is TLR7, encoded by the TLR7 gene located on the short arm of the X chromosome (Kemball et al., 2010; Kutikhin, 2011). The TLR signaling pathway is expressed in both the tumor and immune cell types, which significant for stimulating immune system responses. so, may be associated with developing forms of malignancies (Singh et al., 2023). Normal cells can turn into cancer cells by mutation or other genetic reasons resulting in loss the normal cell cycle (Al-mosawy et al., 2020). Breast cancer is an extremely significant public health problem that affects many women worldwide (Abdulridha Al-Ganimi & Abd Al-Salam, 2023). Breast cancer (BC) is a complex disease due to its diverse morphological features, variable clinical outcomes, and response to different therapeutic options (*KHALID*, 2017). The risk factors of this cancer include aging, reproductive patterns, history of menarche, breast features, hormone use, tobacco and alcohol consumption, body routines, and diet. In the early stages, there are few indications or symptoms, so early detection is an essential method to improve outcomes (Fillatreau et al., 2021). Early detection of breast cancer leads to longer-term reductions in mortality rates, so detecting cancer cells in their early stages is essential for a better prognosis. Increased data suggests a significant relationship between TLRs and breast cancer progression (Singh et al., 2023). Breast cancer genesis and progression is a complicated, multi-step process involving many genetic and epigenetic changes. External environmental variables and internal cellular microenvironmental cues impact the development of the alterations that cause tumorigenesis (Thakur et al., 2022). The study aimed to shed light on the relationship between TLR7 gene polymorphisms and serum levels of biochemical markers [Cancer Antigen 15-3) CA 15-3), Carcinoembryonic Antigen (CEA), Cancer Antigen 125 (CA125), and Cancer Antigen 27- 29 (CA 27-29)] in Iraqi women with breast cancer.

2. Material, Patients and Method

2.1. Patients

This study included 100 women volunteers: 50 with breast cancer as a patient group and 50 who appeared to be healthy as a control group. The ages of all participants were ranged between 29 to 75 years.

2.2. Study Design

The present study was designed as a Case-Control study. The study design has two parts: the first part is related to the biochemical study and includes: biochemical assays [Cancer Antigen 15-3 (CA15-3), Carcinoembryonic Antigen (CEA), Cancer Antigen 125 (CA125), and Cancer Antigen 27- 29 (CA 27-29)] while the second part is related to the genetic study and includes: DNA Extraction, agarose gel electrophoresis, primer desion, PCR, and sequencing. This study was conducted from November 2022 to April 2024.

2.3. Ethics Approval and Consent to Participate

This study was authorized by the Institutional Ethics Committee of Clinical Laboratories, College of Applied Medical Sciences, University of Kerbala, (IQ.UOK.CAMS.DCL.REC.1). Each patient provided informed permission in their

original language, indicating their desire to participate in the study. Patient confidentiality was preserved throughout the research process.

2.4. Blood Collection

The samples of blood were collected from the volunteers at the Al-Imam Al-Hussein Center for Oncology and Hematology in Karbala, Iraq. Five milliliters (5ml) of the venous blood sample from each participant was obtained and separated into two parts: two milliliters (2ml) in EDTA tubes for DNA extraction and three milliliters (3ml) in gel tubes for biochemical tests. Disposable syringes were used to draw the blood in sterile conditions. The blood collected in gel tubes was centrifuged to extract serum, which was subsequently used in biochemical assays [Cancer Antigen 15-3 (CA 15-3), Carcinoembryonic Antigen (CEA), Cancer Antigen 125 (CA125) and Cancer Antigen 27- 29 (CA 27-29)] using enzyme-linked immunosorbent assay (ELISA) kits. Special research kits (sandwich ELISA kits) for these four biochemical markers from BT LAB Company, China were used.

2.5. DNA Extraction Kit

In order to extract DNA from the obtained blood, the EDTA tube was frozen. The ReliaPrep™ Blood gDNA Miniprep System (Promega Company, USA) used to extract DNA from blood samples. The DNA extraction kit consists of Binding Buffer (BBA), Cell Lysis Buffer (CLD), Collection Tubes (2ml), Column Wash Solution (CWD), Nuclease-Free Water, Proteinase K Solution (PK), and Binding Columns. It was used to isolate 100 genomic DNA isolates from 300 µl of the whole blood for each sample.

2.6. Molecular Detection

The total volume used in PCR reaction in this study was 25 µl and included 5 µl DNA, 2 µl from each primer, 8 µl nuclease-free water, and 8 µl master mix (a ready-to-use mixture of Easy Taq DNA Polymerase, dNTPs, and optimized buffer) from Promega Company, USA as described in Table 1. The target sequence of the Intron 2 region in the TLR7 gene was amplified using a specific PCR program as shown in Table 2.

Table 1: Primers Designed for TLR7 Gene Amplification in the Intron 2 Region of this Study

Primer name		Primer sequence	PCR product size
Intron 2	*TLR7*-Forward	5'-CATGGTGATGATGACAGCAA-3'	580 base pair
	TLR7-Reverse	5'-GGCCACTCAAGGACAGAACT-3'	

Table 2: The PCR Program.

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

The PCR product bands were separated using agarose gel electrophoresis on a 1% agarose gel dyed with fluorescent Red Safe dye (Promega Company, USA). The gel electrophoresis system was set for 60 minutes at 70 volts, an UV transilluminator was used to see the gel Fig.1. The Primer3Plus bioinformatics program was utilized to design the primers

(Forward and Reverse Primer for the Intron 2 region of TLR7 Gene) for this investigation (Figure 2; Figure 3). The primers were ordered from Alpha DAN, S.E.N.C. in (Montreal, Quebec, Canada), and synthesized on automated computer-controlled synthesizers using standard phosphonamidite chemistry.

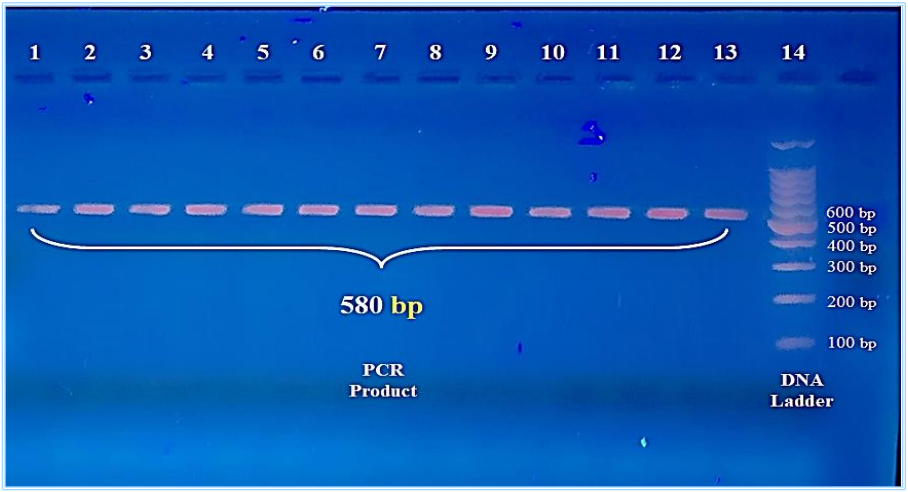

Figure 1: PCR Products (580 Base Pair) Detected in Study Samples, Indicating That the Target Region (Intron 2) Is Present In The TLR7 Gene (The Mentioned Figure Explain And Shows Our Study PCR Products Results)

2.7. Nucleotides Sequencing and Analysis:

PCR products for 20 samples of the patient group were sent to Alpha DNA (S.E.N.C.) Corporation in Montreal, Quebec, Canada, for nucleotide sequencing. A sequencing data were manually analyzed using bioinformatics tools, compared to human reference gene sequences that had already been uploaded to the National Center for Biotechnology Information (NCBI). A basic Local Alignment Search Tool (BLAST), one tool of NCBI, is used to perform alignments Fig.2. Molecular Evolutionary Genetics Analysis X (MEGAX) was employed to examine the sequenced region of the target gene. Multiple sequence alignments were carried out using the CLUSTALW program to verify existence variants identified by the BLAST tool. The variants' location in sequenced DNA samples found in the current study were reported and examined using tools from the Ensemble Genome Browser tools to identify the type of variant and forecast the functional implications Fig.3. The previously described browser was also used to identify new (novel) variations found in this study.

2.8. Statistical Analysis

Statistical analysis was carried out using SPSS version 22.0 (SPSS, IBM Company, Chicago, IL, USA). Data was expressed as means ± standard error (SE) normally distributed and were compared using the independent samples T-test. Data was expressed as a median ± Interquartile range (IQR) if non-normal distributed and were compared using the Mann-Whitney U test. The P-values ≤ 0.05 were considered statistically significant differences. The Kruskal-Wallis test and one-way analysis of variance were also used. Cohen's D describe the effect size used to indicate the standardized difference between the two means. Also, use the point biserial correlation coefficient (r_{pb}).

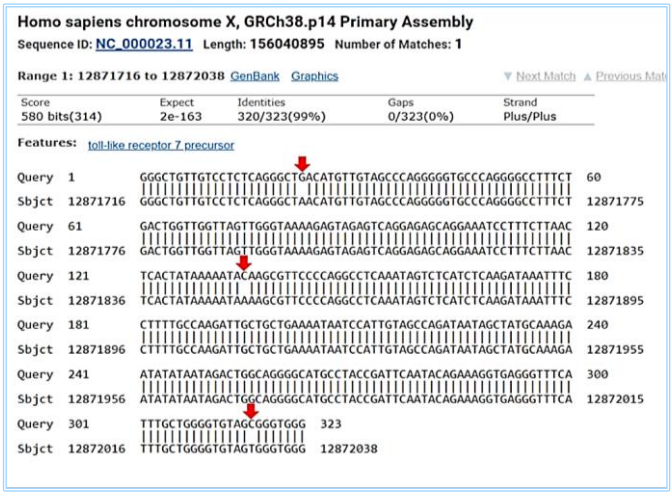

Figure 2: Alignment of Sequence by The BLAST Tool
(The Mentioned Figure Explain and Shows Our Study PCR Products Results)

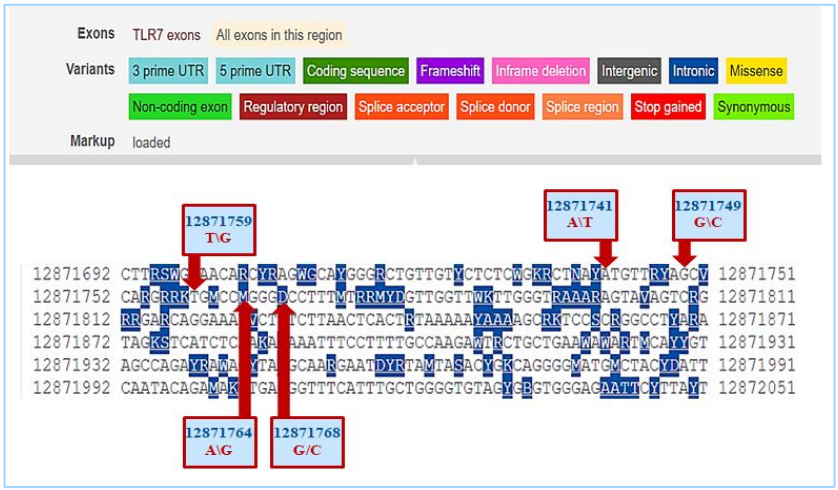

Figure 3: The Molecular Location of Newly Detected, Non-Registered Variations in Study Samples

3. Results

The results of this study showed that there were statistically significant differences when the serum levels of the four tumor markers namely, CEA, CA 125, CA 15-3, and CA 27-29 were compared between control and patient groups ($p<0.001$). The calculated Cohen's D for the mentioned four comparisons of -0.861, -0.753, -0.705, and -1.021 suggests a large-sized effect, indicating that there was a practical and meaningful difference in the mean serum levels of the four tumor markers under study. The advantage was in favor of the patient group, highlighting it was potential significance for the differentiation between controls and patients Table 3.

Table 3: The Description of Mean Values for Biochemical Markers by Using Cohen's D (N=100).

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

Conventional PCR used to amplify the target region of DNA (the Intron 2 region of the TLR7 gene), which includes (580 base pair). PCR products (580 bp) of the Intron 2 region were detected in all study samples, indicating the presence of the target regions in the TLR7 gene. The genetic analysis of the 20 samples showed that the 6 out of the 20 samples demonstrated double variations Table 4, and the rest of the 14 samples displayed multiple variations Table 5 in their intron 2 region sequences Fig.3 in the methods section.

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

Sample#	Number of Variations	Variants Location			
5	2	A\G	12871738	A\T	12871741
9		A\T	12871741	G\C	12871749
14		A\G	12871738	A\C	12871850
17		T\G	12871759	T\A	12871888
19		A\G	12871738	A\C	12871850
20		A\G	12871738	A\C	12871850

Table 5: Multiple Variants on Intron2 Region Appeared in the Study Samples

Sample#	Number of Variations	Variants Location					
1	3	A\G	12871738	G\C	12871749	A\T	12871741
2	5	A\G	12871738	G\C	12871768	G\C	12871749
		A\T	12871741	A\C	12871850		
3	7	A\C	12871850	T\G	12871759	G\C	12871749
		A\G	12871738	A\G	12871764	G\C	12871768
		A\T	12871741				
4	3	A\G	12871738	G\C	12871749	A\T	12871741
6	5	A\G	12871738	A\G	12871764	T\G	12871759
		A\T	12871741	T\A	12871888		
7	6	A\G	12871738	A\G	12871764	G\C	12871749
		A\T	12871741	G\C	12871768	A\C	12871850
8	3	G\C	12871749	T\A	12871888	T\G	12871759
10	4	A\T	12871741	G\C	12871749	T\G	12871759
		G\C	12871768				
11	5	A\G	12871738	A\G	12871764	T\G	12871759
		G\C	12871749	A\C	12871850		
12	7	T\G	12871759	G\C	12871749	A\T	12871741
		A\G	12871764	A\C	12871850	T\A	12871888
		A\G	12871738				
13	4	A\G	12871738	A\G	12871764	A\C	12871850
		G\C	12871749				
15	4	A\G	12871738	T\G	12871759	A\C	12871850
		G\C	12871749				
16	5	A\G	12871738	A\C	12871850	G\C	12871768
		T\G	12871759	T\A	12871888		
18	6	G\C	12871768	A\G	12871764	T\G	12871759
		A\G	12871738	A\C	12871850	T\A	12871888

The genetic analysis detected Five novel non-registered variants in the Intron 2 region: [12871741 A\T, 12871749 G\C, 12871759 T\G, 12871764 A\G, 12871768 G\C] Table 6; Fig.3 in the. methods section

Table 6: Novel Non-Registered Variations Detected in the Study Samples.

Region	Variants Location	Allele	Sample#	Total
Intron2	12871759	T\G	3,6,8,10,11,12,15,16,17,18	10
	12871749	G\C	1,2,3,7,8,9,10,11,12,13,15	11
	12871741	A\T	1,2,3,5,6,7,9,10,12	9
	12871764	A\G	3,6,7,11,12,13,18	7
	12871768	G\C	2,3,7,10,16,18	6

The effect of TLR7 gene variants on biochemical markers CEA, CA125, CA15-3, and CA27-29 was studied. This was done by comparing the levels of each biochemical marker in the samples pooled with a number of mutations. The results of the samples were divided into three categories according to the number of mutations in each sample. The first category includes the samples that contain fewer than 3 mutations, the second category includes the samples that contain 3 to 5 mutations, and the third category includes the samples that contain more than 5 mutations. The results showed the effect of TLR7 gene variations on serum levels of CEA, CA125, CA15-3, and CA27-29 in the Intron 2 region. Non-significant differences between the three categories (<3, 3-5, and >5 mutations) in the level of CEA, CA 125, CA 15-3, and CA 27-29 with p-value 0.56, 0.89, 0.14, and 0.69, respectively Table 7.

Table 7: Comparison of Biochemical Markers Between Different Groups for The Intron 2 Region (Three Groups Divided Based on The Number of Mutations In Each Sample).

No. mutations	Total Samples	CEA (Median±IQR)	CA 125 (Median±IQR)	CA 15-3 (Median±IQR)	CA 27-29 (Mean±SE)
<3	9	271.26±162.4	11.2±14.8	65.3±24.4	45.59±6.67
3-5	7	276.2±97.2	11.1±2.6	112.5±218.7	38.44±8.85
>5	4	339.7±170.0	21.3±24.0	93.5±187.2	49.0±9.35
P-value		0.56	0.89	0.14	0.69
The results indicated a non-significant difference by using the Kruskal-Wallis test, and one-way ANOVA					

The result of samples was divided into three groups based on the status of variations present (either previously registered variations or new, non-registered variations). The first group (New) included samples that contained new, non-registered (novel) variations only, the second group (Mixed) included samples that contained previously registered variations and new, non-registered variations, while the third group includes previously registered variations only. The result showed non-significant differences between the new, mixed, and old variations in the level of CEA, CA125, CA15-3, and CA27-29, with a p-value 0.64, 0.87, 0.35, and 0.92, respectively Table 8.

Table 8: Differences in Mean Values of Biochemical Markers According to Different Variations in Intron (N=20)

Variations status	N.	CEA (Mean±SE)	CA 125 (Median±IQR)	CA 15-3 (Median±IQR)	CA 27-29 (Mean±SE)
New	2	271.0±17.81	11.12±0	81.94±0	43.29±18.19
Mixed	15	293.95±27.81	11.62±20.5	81.77±52.5	44.76±5.9
Old	3	356.5±96.45	11.15±0	58.82±0	39.15±4.61
P-value		0.64	0.87	0.35	0.92
The results indicated no significant difference by Kruskal-Wallis test and one-way ANOVA					

A point biserial correlation coefficient (r_{pb}) was conducted to examine the relationship between each SNP (Single Nucleotide Polymorphism) with the four biochemical markers (CEA, CA125, CA15-3, CA27-29). SNP A/G (12871764) of the Intron 2 region was moderately associated with the CA 15-3 biochemical marker levels (point biserial correlation coefficient = -0.385), and the two variables were statistically significantly associated (p = 0.036). SNP G/C (12871749) of the Intron 2 region was moderately negatively and statistically significantly correlated with the CEA biochemical marker serum levels (point biserial correlation coefficient = -0.396, p = 0.03).

4. Discussion

TLR7 gene polymorphism significance for oncogenomic (a sub-field of genomics that characterizes cancer-associated genes) remains unclear. The TLR7 gene contains significant functional polymorphisms that may impact not only the malignant susceptibility but may also affect responsiveness to therapy. When TLR7 is activated, it promotes B-cell differentiation, which increases the production of autoantibodies and stimulates the production of type I interferon and cytokines, enhancing the immune response (Al-Humairi et al., 2019; Fillatreau et al., 2021).

TLR7 stimulation in tumor-bearing hosts activates antitumoral immunity that can improve disease outcomes in numerous malignancies (Spinetti et al., 2016). Other studies have revealed that TLR7 can induce apoptosis in certain cancer cells, playing an antitumor role. TLR 7 agonists activate innate immune cells, humoral and cellular immunity, and thus produce anti-tumor activities (Chi et al., 2017). TLR7 can be used as novel diagnostic biomarkers, progression and prognostic indicators, and immunotherapeutic targets for cancer. TLR7 agonists have been investigated as possible treatments for immune therapy that targets tumors (Sun et al., 2022).

On the other hand, multiple studies support the notion that TLRs are cancer activators. The expression of TLRs in cancer cells indicates that TLR-mediated signaling is important in cancer tumor progression. TLRs are extensively expressed in breast cancer cells, and activation of these receptors can result in cancer cell proliferation, invasion, migration, and metastasis. Furthermore, When TLR binds to cancer cells, it leads to increased production of immune-suppressive cytokines (Singh et al., 2023), suggesting that tumor cells may use TLR activation to escape from tumor immune surveillance (Kidd et al., 2013; Semlali et al., 2017). The significance of multiple TLR expression in tumor cells is not completely unknown, so more research is needed into the gene variety of TLRs in cancer. Few previous studies were done about the relationship and effect of TLR7 gene polymorphisms on breast cancer. Among the previously registered TLR7 gene variants that were discovered in this study is: rs179019 (X:12871850 A\C), which no previous studies revealed an association between this variant and breast cancer, But it has been significantly associated with other diseases, such as the study by (Ranjan & Panda, 2023) that showed a significant association between the rs179019 variant and susceptibility to SLE development. This association might be explained by the overproduction of pro-inflammatory cytokines in addition to type I Interferon. Also, the rs179020 (X:12871738 A\G), associated with some diseases has been discovered, but its relationship to breast cancer has not been proven, such as the study by (Traks et al., 2015) that showed a significant association between the rs179020 variant and a vitiligo disease (Galimova et al., 2017) in a study on psoriasis disease, revealed a significant association between the risk of developing this disease with the rs179020 variant (Al-Humairi et al., 2019) showed a significant association between this variant with a potential risk for urinary bladder cancer (UBC).The results presented from this study highlighted the importance of the Intron 2 region in the TLR7 gene by detecting novel variants that may affect on the breast cancer prognosis and response to treatment. Notably, five novel variants found in the *TLR7* gene's Intron 2 region (12871741 A\T, 12871749 G\C, 12871759 T\G, 12871764 A\G, 12871768 G\C), may become more significant in the field of *TLR7* gene studies because these mutations exist in a crucial coding region. To precisely identify their role in breast cancer patients, further studies are needed. It must be noted that the results of this study were limited by the relatively small sample size of patients and controls, suggesting the need for large-scale studies to corroborate the results and validate the findings.

Conclusions

Based on the results of the present study, the study found a significant effect of *TLR7*- 12871749 G\C variant of the Intron region on serum level of CEA, a significant effect of *TLR7*- 12871764 A\G variant of the Intron region on serum level of CA 15-3 and the two variants, 12871749 and 12871764, might be the most important variants among the five novel variants.

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