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The Potential Role of *Toxoplasma Gondii* Infection in Breast Cancer Through Affecting Programmed Death-1 Genes

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ABSTRACT

Background: This study aimed to evaluate the possible effects of *Toxoplasma gondii* infection on breast cancer through affecting the serum and expression gene level of programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1) in breast cancer patients from Thi-Qar province, Iraq.

Methods: This case-control study involved 150 patients suffering from breast cancer (BC) who referred to general hospitals of Thi-Qar province between July to September 2023 and 150 healthy patients. An enzyme-linked immunosorbent test (ELISA) kit was used to evaluate the anti-*Toxoplasma* IgG and the serum level of PD-1 and PDL-1. Furthermore, the expression level of PD-1 and PDL-1 was measured through Real-time PCR.

Results: Among 150 breast cancer patients, 71 patients (47.3%) and 25 (16.6%) of healthy subjects exhibited seropositivity for anti-*T. gondii* IgG antibodies, respectively. We found that the serum level and the gene expression level of PD-1 and PDL-1 were significantly higher among BC patients seropositive for *T. gondii* compared with BC patients who were seronegative for *T. gondii* antibodies ($P<0.05$).

Conclusion: These results indicated that *T. gondii* may play an important role in the occurrence and even progression of cancer probably by elevating the levels of PD-1 and PDL-1 genes. However, further studies are required to confirm these findings.

INTRODUCTION

Toxoplasmosis (TS) is a prevalent parasitic infection that affects both humans and animals, caused by *Toxoplasma gondii*.[1] TS affects around one-third of the humans worldwide.[1] This parasite can infect a wide range of warm-blooded animals; however, it can only undergo sexual reproduction in felids, specifically in cats, which are the definitive hosts. The prevalence of *T. gondii* antibodies differs significantly across various regions globally. This parasite is especially common in countries in Western Europe, South America, and Africa.[2]

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The transmission of *T. gondii* commonly occurs through the ingestion of contaminated food and water containing oocysts excreted by cats, or via the consumption of raw or undercooked meat which contains tissue cysts.[2] TS is characterized by an infection that can be acquired, congenital, or inherited, and can result in severe symptoms in individuals with compromised immune systems.[3] Acquired TS is typically asymptomatic and self-resolving in healthy individuals. However, in immunocompromised individuals, such as those with acquired immunodeficiency syndrome (AIDS), the infection can progress to toxoplasmic encephalitis, a severe and potentially life-threatening condition.[3] The primary clinical features of congenital toxoplasmosis include chorioretinitis, cerebral calcification, hydrocephalus, microcephaly, and mental retardation.[3,4]

Breast cancer is the most prevalent malignancy among women globally, representing 23% of the 1.1 million new cancer cases diagnosed in women annually.[5] It is also the main cause of cancer-related deaths worldwide, with higher mortality observed in low-resource countries.[5] Several research studies have shown a notably higher prevalence of toxoplasmosis in cancer patients, particularly those with breast cancer, compared to individuals without cancer.[6]

Programmed death-ligand 1 (PD-L1) is a protein with immunosuppressive properties that hinders T cell activity by binding to the inhibitory receptor programmed death-1 (PD-1).[7] Elevated levels of PD-L1 in cancer cells contribute to immune evasion and are associated with poorer survival outcomes and prognosis in various cancers, including breast cancer.[8] PD-1 and its corresponding ligands, particularly PD-L1, play a crucial role in activating T cells, promoting their proliferation, and facilitating the release of cytotoxic substances. This process contributes to the suppression of anti-tumor immune reactions.[8] *T. gondii* can cause a strong Th1 immune response in host cells, upregulating the expression of interleukin-12 (IL-12) and interferon-γ (IFN-γ).[9] On the other hand, IFN-γ is essential for programmed death ligand-1-mediated T cell stimulation;[10] therefore, we aimed to evaluate the seromolecular prevalence of *T.gondii* infection in breast cancer patients from Thi-Qar province, Iraq and assess the serum and gene expression level of PD-1 and PDL-1 in patients with or without toxoplasmosis.

METHODS

Study area

The province of Thi-Qar is located in the southern region of Iraq at coordinates 31°14'N 46°19'E, with Nasiriyah serving as its administrative center. Covering an area of around 13,000 square kilometers, Thi-Qar is inhabited by a population of nearly 2 million people. The climate of this area is defined by arid and desertic conditions, featuring limited annual rainfall.

Study design and participants

This case-control study involved 150 patients suffering from breast cancer (BC) who referred to general hospitals in Thi-Qar province between July to September 2023 (Figure 1).

Based on the prevalence of IgG positivity related to toxoplasmosis in both BC and healthy groups, as reported in the study by Al-Muskakeh *et al.* (2022)[19], a sample size of 150 individuals was determined for each group. The case and control groups were matched according to the age and residence. The diagnosis of breast cancer was based on clinical examinations confirmed by laboratory tests conducted by relevant specialist doctors. The control group involved 150 healthy individuals with no cancer, who referred to the studied hospitals to do the routine laboratory experiments during the study time. Participants who had taken systemic antibiotics within the past three months, immunocompromised patients, as well as participants who had received intravenous immunoglobulin therapy or immunotherapy before blood collection were excluded.

Questionnaire and risk factors

Prior to data collection, all the participants or their authorized representatives provided informed consent through both verbal and written means. Participants completed a questionnaire pertaining to demographic variables such a, age, and place of residence as well as some *T. gondii* related risk factors such as agriculture activity and consumption of raw or uncooked meat.

Figure 1. The geographical characteristics of the province where the study was conducted.

Sampling

Blood samples were acquired from the participants by drawing five milliliters of blood via sterile vein puncture with disposable plastic syringes. The blood was then placed in a gel tube and left to coagulate at ambient temperature for one hour. Following coagulation, the blood underwent centrifugation at a speed of 4000 rpm for a duration of 10 minutes. The resulting serum was split into two equal parts and transferred to Eppendorf tubes for subsequent immunological assessments before being stored at -20 C°.

Enzyme-linked immunosorbent assay (ELISA) for anti-Toxoplasma antibodies

To assess anti-*T. gondii* antibodies, ELISA kits for *Toxoplasma* and IgG (cAMP, Romania) were used to test all the collected sera according to the manufacturer's instructions.

Determining the serum level of interferon-gamma

The serum level of PD-1 and PD-L1 were measured using a specific enzyme-linked immunosorbent test (ELISA) kit sourced from Sunlong Company, China. The assays were performed in accordance with the manufacturer's instructions.

Evaluating the gene expression by Quantitative Real-Time PCR

Total RNA was extracted from the peripheral blood by the kit procedure from Qiagen, Germany, and the quality of the extracted RNA was evaluated using a Nanodrop from Biotek Epoch. The sample was then converted to cDNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Sankt Leon-Rot, Germany) according to the manufacturer's instructions. Quantitative Real-Time PCR was performed by the SYBR Green master mix 2X (Thermo Fisher Scientific, Heiligen, Germany) using primer designed [11] for the PDL-1 gene forward 5′-TGTGAAAGTCAATGCCCCAT-3′ and reverse: 5′-TGTCAGTTCATGTTCAGAGGT-3′, the PD-1 gene forward 5′- CCAAGGCGCAGATCAAAGAGA-3′ and reverse: 5′- TGGGCTGTGGGCACTT-3′ by denaturation at 95 ℃ for 10 min, followed by 40 extension cycles and a final cycle at 95 ℃ for 15 s and annealing and extension at 60 °C for 30 s. The $2^{-\Delta\Delta Ct}$ value was calculated using Bio-Rad iQ5 Optical System Software (USA) to determine the gene expression levels. The quantity of mRNA was assessed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference for quantifying gene expression (Forward: 5'-AACTTTGGCATTGTGGAAGG-3' and Reverse: 5'-ACACATTGGGGGTAGGAACA-3'). [11]

Statistical analysis

The statistical analysis was done using the SPSS 24.0 software (SPSS Inc., Chicago, IL, USA). Chi-square tests were used to determine the difference in distribution of participants among case and control participants and the associations between infection and studied factors. Since breast cancer is more common in people over 30 years old, we classified the participants' age groups into <34, 34-55, and <55 years. Variables significantly related to *Toxoplasma* prevalence were analyzed as potential risk factors by means of univariate logistic regression. Variables showing a P-value of less than or equal to 0.20 were included as inputs in the final multivariate logistic regression model. In order to determine the chance of exposure to *Toxoplasma* parasite in the patients with breast cancer compared to healthy subjects, the odds ratio with a confidence level of 95% was used. P-values of <0.05 for associations were considered to indicate statistical significance. Furthermore, to compare the levels of PD1 and PDL-1 among all the groups studied and pairwise (two by two between groups), the One-Way ANOVA and Tukey's post hoc test were used, respectively.

RESULTS

Participants

In the present case-control investigation, totally, 300 participants including 150 patients with breast cancer and 150 healthy individuals referred to general hospitals of Thi-Qar province, Iraq, were studied. The mean age of the participants in the BC and non-BC groups was 42.6 and 45.3 years, respectively. In terms of residence, 79 (52.60%) and 86 (57.3%) participants in the in the BC and non-BC groups lived in urban areas, respectively, and the rest lived in rural parts.

Prevalence of anti-T. gondii antibodies

Among 150 BC patients, 71 patients (47.3%) exhibited seropositivity for anti-*T. gondii* IgG antibodies, whereas 25 (16.6%) of the samples collected from the non-BC group of healthy subjects demonstrated the presence of anti-*T. gondii* IgG antibodies. Univariate logistic regression indicated a statistically significant difference in the prevalence of anti-*T. gondii* IgG antibodies between BC and non-BC groups (P<0.001); this indicated that the likelihood of *Toxoplasma* infection is significantly higher in breast cancer patients compared to those without breast cancer (P<0.001, OR=4.49, 95% CI: 2.63-7.68).

Associated risk factors for T. gondii infection

In the examination of age-related subcategories, there was no statistically significant correlation discovered between the prevalence of *T. gondii*

antibodies and the age of individuals in both the BC (P=0.581) and non-BC (P=0.696) groups. Additionally, a significant association was found between the participants' residential location and the prevalence of *T. gondii* antibodies in the BC (P=0.003) and non-BC participants (P=0.006). In relation to the agricultural activities of the participants, a significant correlation was observed between the agricultural activities and the prevalence of *T. gondii* antibodies in the BC (P=0.047); but no significant correlation was observed between the agricultural activities and the prevalence of *T. gondii* antibodies in non-BC participants (P=0.653). In terms of consumption of raw or uncooked meat, a significant association was found between this risk factor and the prevalence of *T. gondii* antibodies in the non-BC group (P=0.002), but no significant connection was observed between the consumption of raw or uncooked meat and the prevalence of *T. gondii* antibodies and BC participants (P=0.236) (Table. 1).

Table 1. Frequency of *Toxoplasma gondii* antibodies in patients with breast cancer (BC) and healthy individuals (Non-BC) based on the demographic characterizations and the related risk factors

Variable	Univariable analysis		Multivariable analysis	
	Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value
T. gondii seropositivity	4.49 (2.63- 7.68)	<0.001*	4.95 (2.78- 8.82)	<0.001*
Age	0.861 (0.507-1.464)	0.581	-	-
Residence	3.076 (1.475-6.415)	0.003*	3.862 (2.032-4.342)	<0.001*
Agricultural activity	1.575 (0.868-2.859)	0.135	-	-
Consumption of raw/ uncooked meat	2.256 (1.204-4.228)	0.011*	2.361 (1.164-4.790)	0.017*

*P<0.05 significant difference.

In the context of multiple logistic regression analysis, it was found that residing in urban areas (P<0.001, OR=3.86, 95% CI: 2.032-4.342) and the consumption of raw or undercooked meat (P=0.014, OR=2.361, 95% CI: 1.164-4.790) were identified as independent risk factors associated with *Toxoplasma* seropositivity. Multiple logistic regression analysis, after being adjusted for age, residence, agriculture activity and consumption of raw/ uncooked meat, showed that the probability of *T. gondii* seropositivity was significantly higher in breast cancer patients (P<0.001, OR=4.95, 95% CI: 2.78-8.82).

Table 2. Multiple logistic regression analysis for evaluating the association between *T. gondii* seropositivity and breast cancer in Iraq

Variable	Breast cancer patients (N=150) Anti-*Toxoplasma* IgG antibody			Healthy control patients (N=150) Anti-*Toxoplasma* IgG antibody			*P-value for comparison of case and control
	Positive No. (%)	Negative No. (%)	P-value	Positive No. (%)	Negative No. (%)	P-value	
Age							
>34 yrs	9 (45.0)	11 (55.0)		4 (22.2)	14 (77.8)		
34- 55 yrs	47 (50.5)	46 (49.5)	0.581	16 (15.7)	86 (84.3)	0.696	<0.001
55< yrs	15 (40.5)	22 (59.5)		5 (16.7)	25 (83.3)		
Residence							
Rural	14 (29.2)	34 (70.8)		4 (6.3)	60 (93.8)		
Urban	57 (55.9)	45 (44.1)	0.003	21 (24.4)	65 (75.6)	0.006	<0.001
Agricultural activity							
No	54 (43.5)	70 (56.5)		19 (15.9)	100 (84.1)		
Yes	17 (65.3)	9 (34.7)	0.135	6 (24.0)	25 (76.0)	0.653	<0.001
Consumption of raw/ uncooked meat							
No	58 (45.3)	70 (54.7)		15 (12.1)	109 (87.9)		<0.001
Yes	13 (59.1)	9 (40.1)	0.011	10 (38.5)	16 (61.5)	0.002	

*P-value from Chi-square test

Evaluating the serum level of PD-1

Figure 2 exhibits the mean of the serum level of PD-1 among BC and non-BC people with or without

seropositivity to *T. gondii* antibodies. The results showed that BC patients who had seropositive for *T. gondii* antibodies exhibited a high level of PD-1 (1181.5±55.24 pg/ml), compared with *T. gondii* seronegative BC patients (1096.3±27.98 pg/ml). The statistical analysis revealed a significant difference (P<0.001) in the PD-1 levels of breast cancer patients with and without seropositivity to *T. gondi* infection.

Evaluating the serum level of PDL-1

As shown in Figure 2, the results showed that BC patients who had seropositive for *T. gondii* antibodies exhibited a high level of PDL-1 (391.8±12.33 pg/ml), compared with *T. gondii* seronegative BC patients (278.5±13.55 pg/ml). The statistical analysis revealed a significant difference (P<0.001) in the PD-1 levels of breast cancer patients with and without seropositivity to *T. gondi* infection.

Evaluating the gene expression of PD-1 and PDL-1 by Quantitative Real-Time PCR

Figure 3 indicates the gene expression levels of PD-1 and PDL-1 among BC and non-BC people with or without seropositivity to *T. gondii* antibodies. The results showed that the gene expression level of PD-1 among BC patients with or without seropositivity to *T. gondii* antibodies was 3.12±0.21 and 2.39±0.18-fold, respectively. In addition, the gene expression level of PDL-1 among BC patients with or without seropositivity to *T. gondii* antibodies was 2.96±0.24 and 2.16±0.19-fold, respectively. The statistical analysis showed that the gene expression level of PDL-1 was significantly higher among BC patients seropositive for *T. gondii* compared with BC patients who were seronegative for *T. gondii* antibodies (P<0.05).

DISCUSSION

Toxoplasmosis typically resolves on its own in people with healthy immune systems, but the parasites can live in the host's body for years as tissue cysts. During this stage, humoral and cellular immune responses by T lymphocytes and macrophages control tissue cysts. [12] Patients with compromised immune systems, particularly those with chronic cellular immune deficiency, and patients with conditions such as cancer, collagen tissue diseases, organ transplant recipients undergoing immunosuppressive therapy, or hemodialysis patients with chronic renal failure, are at a higher risk of being infected with toxoplasmosis. [12] *T. gondii*, exerts influence over various signaling pathways to ensure its survival within host cells and is significantly linked to immune and inflammatory responses. [13]

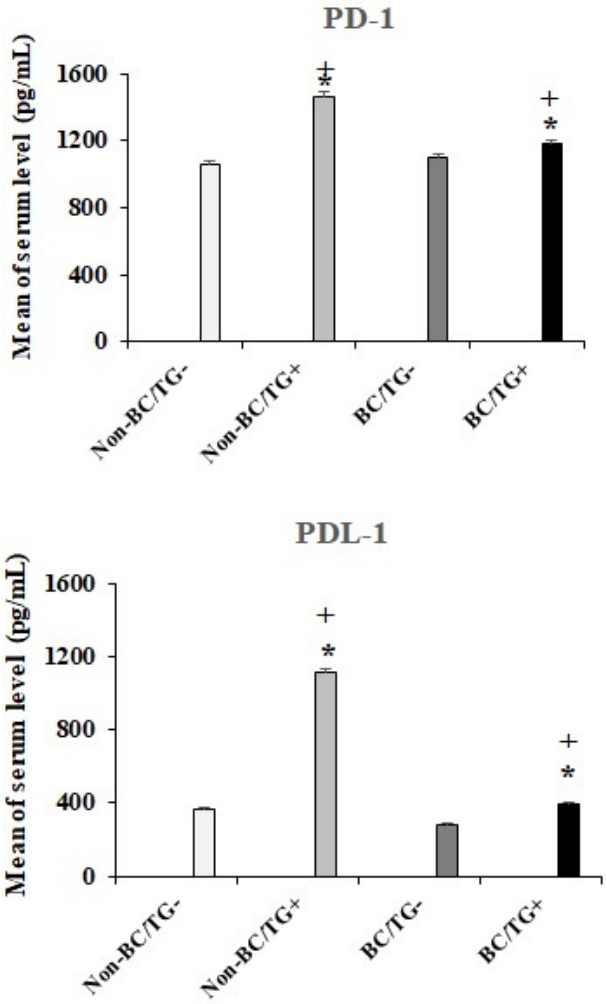

Figure 2. The mean of the serum level of Programmed death-ligand 1 (PD-L1) and programmed death-1 (PD-1) among breast cancer patients (BC) and healthy individuals (non-BC) with or without seropositivity to *T. gondii* antibodies (TG) by ELISA method. Data are indicated as Mean±SD. * P< 0.05 significant difference compared with non-BC/TG-; + P< 0.05 significant difference compared with BC/TG-.

The infection caused by *T. gondii* has the capacity to alter specific host signaling pathways, which are frequently disrupted in the context of carcinogenesis. On the other hand, *T. gondii* may also affect tumor proliferation due to its potent immune-stimulatory properties. Recent research has indicated that *T. gondii* infection, which leads to chronic inflammation in tissues, could play a role in cancer development by modulating essential host signaling pathways, or it may demonstrate anti-tumoral effects. [13]

The present study showed that *T. gondii IgG* antibody was found in 47.3% of breast cancer patients. In line with our results, a study conducted by Azab Hameed and Khalaf (2024) reported that 60 (85.7%) of the patients were positive to *T. gondii* IgG antibody compared to 10 (14.2%) of the patients who were negative for *T. gondii* IgG antibody.[14]

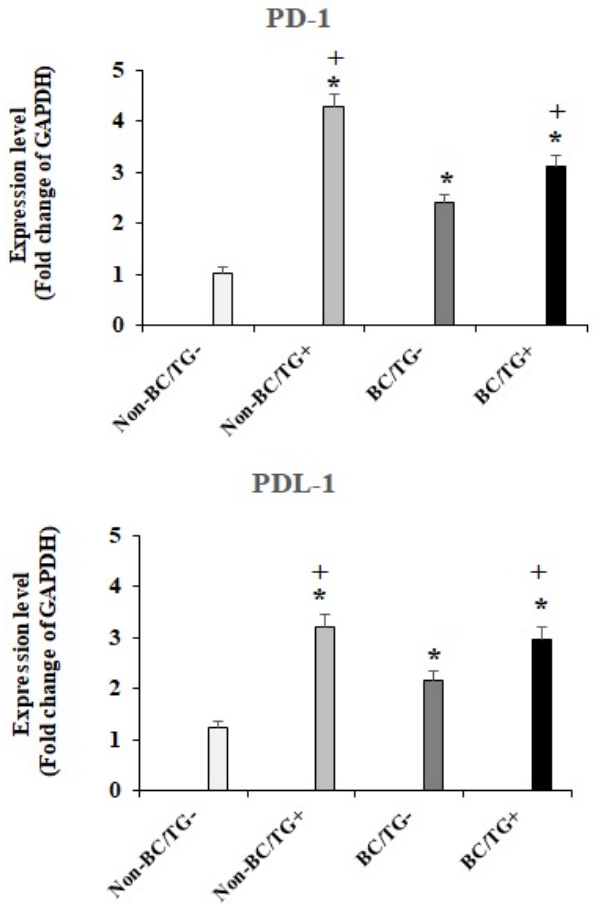

Figure 3. The gene expression level of Programmed death-ligand 1 (PD-L1) and programmed death-1 (PD-1) among breast cancer patients (BC) and healthy individuals (non-BC) with or without seropositivity to *T. gondii* antibodies (TG) by ELISA method. Data are indicated as Mean±SD. *P<0.05 significant difference compared with non-BC/TG-; +P<0.05 significant difference compared with BC/TG-

The protein PD-1, also referred to as CD279, is produced in humans through the PDCD1 gene. PD-1 is a receptor found on the surface of T cells and B cells, comprising 288 amino acids, and is involved in modulating the immune response towards human body cells. [15, 16] Conversely, PD-L1, also known as CD274, is a protein encoded by the CD274 gene in humans. It is a 40kDa transmembrane protein of type 1, believed to have a significant role in suppressing the adaptive immune response in specific circumstances such as autoimmune diseases and other pathological conditions. [17] The PD-1/PD-L1 pathway has been identified as a critical factor in enabling cancer cells to evade immune detection. This pathway involves the expression of PD-1 on both effector T-cells and exhausted T-cells within the tumor microenvironment (TME), as well as the expression of PD-L1 on the cell surface in various cancer types such as lung, bladder, colon, breast, kidney, ovary, cervix, melanoma, glioblastoma, multiple myeloma, and T-cell lymphoma. [18]

Here, we evaluated the serum and expression gene level of PD-1 and PDL-1 in breast cancer patients with or without toxoplasmosis. Our results revealed that BC patients who were seropositive for *T. gondii* antibodies exhibited a high serum gene expression level of PD-1 and PDL-1, compared with *T. gondii* seronegative BC patients. Consistent with our results, a study conducted Al-Muskakeh *et al.* (2022) in Babil City showed that the *T. gondii* infected patients had significantly higher serum levels of PD-1 and PDL-1 than the negative control group. [19] Another study conducted by Sadoon and Khalaf (2023) showed that the serum level of PD1 and PD-L1 was significantly elevated in diabetic patients seropositive for *T. gondii* antibodies. [20] Additionally, Atwan *et al.* (2021) reported that there was an increased level of PD1 and PD-L1 in the placental of women with toxoplasmosis. [21]

Elevated levels of PD-1 and PD-L1 may suggest a resurgence of latent infection as *T. gondii* can trigger CD8 T-cell exhaustion, leading to diminished cellular functionality or reduced production of cytokines and cytotoxic agents by T cells. Furthermore, exhausted T cells exhibit heightened expression of PD-1 and other inhibitory receptors like PD-L1, which impede T-cell activation and contribute to enhanced programmed cell death of lymphocytes. Furthermore, PD-1 signaling modulates T-cell function, resulting in heightened lipolysis and lipid utilization. [22, 23]

The precise mechanisms through which T. gondii triggers carcinogenesis remain unclear. Studies have suggested that *T. gondii* may transfer miRNAs to host cells, potentially influencing host gene expression and contributing to cancer development. [24] Research has shown that *T. gondii* infection can induce and advance brain cancer by altering host miRNA levels. [25] The dysregulation of miRNAs that target both pro-apoptotic and anti-apoptotic genes can disrupt the balance between apoptosis-inducing and anti-apoptosis pathways, ultimately impacting the fate of the host cell's apoptosis process. [26] Conversely, previous research results indicate that infection with *T. gondii* could potentially impact the survival rates of individuals with breast cancer by regulating signaling pathways associated with cytokines. These investigations propose that *T. gondii* infection may enhance the prognosis of breast cancer patients, with the extent of this improvement being contingent upon the levels of certain serum cytokines, notably IL-17 and IL-9. [27-29] The main limitation of this study includes the limited sample size; Other limitations include our failure to analyze the tissue expression of the tested genes, lack of tissue biopsy analysis for parasite presence, uncertainty of the exact time to start breast cancer treatment for adjusting with

toxoplasmosis, which are planned for investigation in subsequent research.

CONCLUSION

The present study reported the high prevalence *T. gondii* antibodies among breast cancer patients in Iraq. We also found that the serum and expression level of PD-1 and PD-L1 was significantly higher among breast cancer patients who were seropositive to *T. gondii* antibodies compared to breast cancer patients who were seronegative to *T. gondii* antibodies. These results show that through patients' follow-up and accurate evaluations, *T. gondii* may play a role in the occurrence and progression of cancer, possibly by increasing the levels of PD-1 and PDL-1 genes. However, further studies are needed to confirm these findings.

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None.

ETHICAL CONSIDERATIONS

Patient enrollment was performed in line with the 1964 Helsinki declaration and the study was reviewed and approved by the ethical committee of University of Thi-qar, Thi-qar, Iraq (No. 2022237).

DATA AVAILABILITY

All data is available in the text of the manuscript and Tables.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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