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Identification of B1 and SAG3 of Toxoplasma Gondii Genes in Breast Cancer Tissues Among Females in Kerbala Province, Iraq

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P A P E R I N F O

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Abstract

The present investigation was done in November 2023. One hundred fifty specimens of malignant Breast cancer tissue, covered with paraffin wax and diagnosed by a specialist, have been gathered along with fifty specimens of benign tumour tissue. The control set was comprised of specimens taken from the histopathologist lab at Imam AL- Hussein Medical City (IHMC), the Al-Kafeel Spec Iality Hospital Lab (KSHL), and the Specialized Al-Sajjad Laboratory (SSL) for histopathology and tumour identification. The investigation contained two sets of specimens. The initial cohort comprised 66 females with breast carcinoma who did not receive chemotherapy, while the subsequent cohort included 84 females with breast carcinoma who did take chemotherapy. PCR technique was utilized on DNA from specimens of tissue afflicted by breast carcinoma across all studied sets, focusing on the amplification of the B1 and SAG3 genes.This work attempted to ascertain the prevalence of toxoplasmosis in patients with breast carcinoma. The toxoplasmosis incidence in patients with breast carcinoma was 11.3%, and the infection rate in normal participants was 4 percent. The data indicated that the infection rate among females who underwent therapy was higher than that of untreated females, attributable to their weakened immunity and increased infection risk, resulting in a 3.7 % greater probability of patients females acquiring breast carcinoma in comparison to normal females.

1. INTRODUCTION

Toxoplasmosis was a zoonotic disease resulting from *Toxoplasma gondii (T.gondii)*, an obligate intracellular protozoan that infects people as well as animals that are warm-blooded as intermediate hosts, with different feline family members serving as intermediate and final parasite hosts [1]. Toxoplasmosis infection is worldwide in its spread among humans, as it varies from one region to another, and about a 3rd the world's population is exposed to infection with the parasite [2]. The infection is often symptomatic in immune-competent persons. However, it can be severe and perilous for those who are immunocompromised, such as pregnant females and those with AIDS. Research identified the potential of the *T.gondii* parasite to induce dysplasia in the reproductive system and aberrant adhesions inside the uterus, leading to infertility in females [3].

Cancer is an abnormal and random growth of cells that originate from a single cell with

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malfunctioning regulatory mechanisms and whose growth exceeds and does not keep pace with the growth of the normal tissue from which it originated. These cells continue to hyperdrive even after the stimulus or stimulus that provoked these changes has disappeared [4].There are many kinds of cancer and the most important one is breast carcinoma; it is one of the most common kinds in females and it has been diagnosed repeatedly among female all over the world. It is considered the second leading to cancer-related deaths [5]. It has been observed that the incidence of the number of infections in Iraq with this kind of cancer has increased in the latest years from 30/100,000 to 40/100,000 in the period between 2006 and 2012 [6]. Breast carcinoma ranks first among the malignant tumors that affect society. In Iraq, it represents 19.5 percent of all cancer cases and 34.3 percent of cancers that affect females. In 2016, more than 897 females died from this disease, which is the first cause of cancer-related deaths among Iraqi females [7]. T.gondii is a possible cause of cancer and it has a function in the development and induction of malignant diseases. This is explained by several theories, including preventing apoptosis and enhancing the movement of dendritic cells and macrophage cells. Another theory detected that *T.gondii* works to accumulate oncogenic mutants due to the disruption of traditional barriers [8]. Studies have proven a potential relationship between malignant and toxoplasmosis diseases, including brain and oral cancers [9]. Furthermore, in Iran, *T. gondii* DNA was discovered in breast carcinoma tissues fixed with paraffin and formalin wax [10]. The genetic and diagnosis description of the *T.gondii* is essential in clinical management, epidemiological investigation, and control of parasites in animals and humans [11]. Polymerase chain reaction (PCR) has been widely utilized in detecting Toxoplasmosis infection since it was first utilized by [12] to target the *B1* gene [13]. PCR also utilizes traditional sequences targeting single-copy genes such as *SAG1*, *SAG2*, *SAG3*, and *GRA1* in humans and animals [14].

2. MATERIALS and METHODS

2.1 Specimens Collection

A total of 150 specimens of aggressive breast carcinoma tissue, embedded in paraffin wax and identified by a specialist, were gathered, along with fifty specimens of benign tumor tissue. They were deployed as a control set. The specimens were obtained from the IHMC, KSHL, and the SSL tissue cutting lab. The investigation comprised two sets of specimens. The initial set comprised 66 females with breast carcinoma who were not given chemotherapy. On the other hand, the second set consisted of 84 females with breast carcinoma who had undergone chemotherapy. PCR technology was utilized on DNA from breast carcinoma-affected tissue specimens across all studied sets, focusing on amplifying the *B1* gene. *SAG3*.

2.2 Genomic and Extraction

Deoxyribonucleic acid was isolated from cancerous breast tissue specimens. Five sections, each 10 microns thick, were removed from tumor tissue of breast encased in paraffin wax to isolate the DNA genomes utilizing the DNA Genomic Mini Kit Tissues Protocol, mainly developed for this procedure. I used the Korean commercial kit manufactured by Favorgen for extracting DNA from cancerous breast tissue specimens.

2.3 The Utilized Primer In PCR

Particular primers for the *B1* gene have been obtained from [15] and for the *SAG3* gene from [16], manufactured by the Korean business Bioneer, as shown in **Table 1**.

TABLE 1. Sequence of primers used in molecular studies

Primers sequence (5`-3`)			PCR product size
Gene *B1*	Primer Forward	GGAACTGCATCCGTTCATGAG	194bp
	Primer Reverse	TCTTTAAAGCGTTCGTGGTC	
Gene *SAG3*	Primer Forward	CGCGACAC AAGCTGCGATAG	1158bp
	Primer Reverse	TTAGGCAGCCACATGCACAA	

The PCR was conducted utilizing a commercial 20µ Reaction kit from the Korean business Addbio, following the manufacturer's instructions (Table 2).

TABLE 2. Components of the Master Blend for PCR

Components	Volume (µL)
Polymerase enzyme Taq DNA Polymerase	1U
Each: d NTP(d ATP, CTP,d GTB,dTTP A mixture of nitrogenous bases	400 µL
Buffer solution	10 µL
Loading dye	30 µL
MgCl2	3 µL

2.4 Preparing the Polymerase Chain Reaction (PCR) Mixture

This combination was created according to the manufacturer's specifications in Table (2). All components listed in the Table have been put in specialized tubes. The tubes have been sealed and subjected to the microcentrifuge at the highest speed for thirty seconds. The tubes have been moved to the thermal cycler of PCR.

TABLE 3. PCR Mixture

PCR Blend	Volume (µL)
Extraction of DNA	2
Primer Forward	2
Reverse Primer	2
Free DNAase water	6.5
Master mixing	12.5
Total volume	25

2.5 PCR Program PCR Thermo cycle

The PCR was conducted applying a thermal cycler from the Chinese business Biobase, resulting in the amplification of the target genes B1 and SAG3 to sizes of 1158 bp and 194 bp, respectively, as illustrated in Tables 4 and 5.

TABLE 4. The *B1* gene utilized program in the PCR machine

Step	Temp (C°)	Duration	Number of Cycles
DNA Initial Denaturation	95	3 min	35
Denaturation		3s	
Annealing	60	30 min	
Extension	72	1	
Final extension		5	

TABLE 5. Thermal cycling program for the *SAG3* gene

Step	Temp (ºC)	Duration	Number of Cycles
DNA Initial Denaturation	95	5 min	35
Denaturation		35s	
Annealing	60	30 min	
Extension	72	55s	
Final extension		5min	

Five microliters from PCR products were visualized utilizing a UV Transilluminator. The remaining 20 microliters from the PCR product were subjected to DNA sequences to identify genetic variation. The favorable PCR *SAG3* gene products were shipped in an ice bag to Macrogen Company in Korea for DNA sequencing on an AB DNA sequencing machine through DHL. Molecular Evolutionary Genetics Analysis version 6.0 was utilized to calculate evolutionary distances by employing the "Maximum Composite Likelihood technique utilizing the phylogenetic Tree (UPGMA) Method and the Multiple Sequence Alignment Analysis of the Incomplete *SAG3* Gene Based on the Clustal W Alignment Analysis" by Utilizing Software (Mega 6.0). The collection of *T.gondii* sequences in Genbank of *T.gondii* sequences was performed utilizing "NCBI (https://www.ddbj.nig.ac.jp/ddbj/updt-form-e.html)" to compare local SAG3 sequences with other global sequences. The main inclusion criterion was linear DNA sequences of the SAG3 marker with a broad geographical representation from different regions with definitive or intermediate hosts of similar size. The size of the *SAG3* marker is 1158 bp. Phylogenetic analysis utilizing multiple sequence alignments, especially when the sequences are not highly conserved, necessitates the removal of poorly matched locations and divergent sections, as these regions are unlikely to be homologous and may be saturated by repeated replacements.

TABLE 6. Investigation sets based on infection

	Investigation sets				Total		X^2p-magnitude	OR (95 percent CI)	RR (95 percent CI)
Toxoplasma. test	patients		Control						
	N	percent	N	percent	N	percent			
Ve+Toxo.	17	11.3percent	2	4.0 percent	19	9.5 percent	2.346 0.126 Ns	3.07 (0.68-3.77)	2.83 (0.68-11.84)
Ve- Toxo.	133	88.7percent	48	96.0 percent	181	90.5 percent		Ref.	

2.6 Sectioning

The wax molds containing the tissues were placed in the microtome manual tissue cutting equipment, with the desired thickness set at about 3 to 5 micrometers, to produce strip sections of the tissue samples. The strips are arranged on a black plate for selection beside 1.5 ml Eppendorf tubes [17].

2.7 Tissue staining

The tissue slices have been stained with hematoxylin-eosin according to the techniques outlined in [18]. The paraffin-embedded and formalin-fixed cancerous breast tissues were sectioned to 4-5 microns, affixed to glass slides, and allowed to dry at normal temp for one day.

2.8 Statistical analysis

The categories of factors are expressed as percentages and numbers and evaluated utilizing Fisher's exact or chi-square tests, as suitable [19], [20]. Continuous factors are expressed as average±standard deviation and evaluated utilizing Student's t-test. Risk variables for breast carcinoma were evaluated applying a model of logistic regression and reported as odds ratios (OR) with corresponding confidence intervals (CI)95 percent. All assessments have been performed utilizing SPSS version 28. Differences with p-magnitudes below 0.05 have been considered statistically significant.

3. RESULTS AND DISCUSSION

This study detected 150 females diagnosed with cancer of the breast, with a mean age ranging from 25 to 56 years. 17 females infected with *T.gondii* have been identified as illustrated in Table 1:

Toxoplasmosis incidence increased to almost 40 percent during the occupation of Iraq, in contrast to 2 percent in the 1980s [21]. In 2016, 335 patients infected with Toxoplasma were documented throughout all Iraqi governorates [22]. The frequency of toxoplasmosis varies for several reasons, including climatic conditions differences and cultural patterns throughout various areas of Iraq [23]. Research in Iraq detected that the prevalence of breast carcinoma among females was 33.81%. Relative to other Arab country, the breast carcinoma incidence was lower in certain countries, including Bahrain, Jordan, and Kuwait.

Conversely, the rate was elevated in other Arab nations, including Qatar, the UAE, Oman, and Saudi Arabia, as well as in bordering Arab countries like Turkey and Iran [24], [25]. Limited information exists on the incidence of T. gondii infection in immunocompromised individuals undergoing neoplastic disease treatment or immunosuppressive therapy in Iraq [26]. Recent research detected that the incidence toxoplasmosis rate among patients with breast carcinoma was 77.50 percent [27]. Chronic inflammation often induces carcinogenesis and can expose a person to cancer.

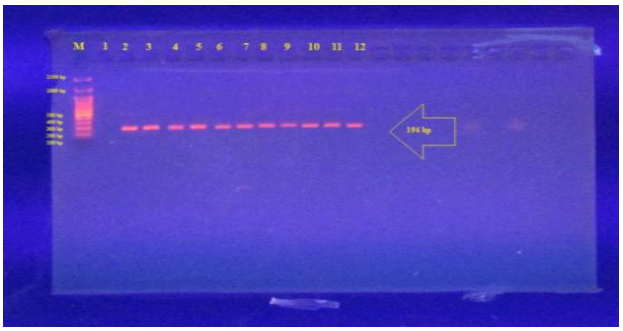

Figure 1. *B1* gene DNA Detection

T.gondii B1 gene PCR product Electrophoresis was conducted on an agarose gel at a voltage=60 for thirty minutes, employing the fluorescent red dye Ethidium Bromide. The specimens exhibited a band corresponding to 194 base pairs of DNA isolated from patients with cancerous breast tissues.

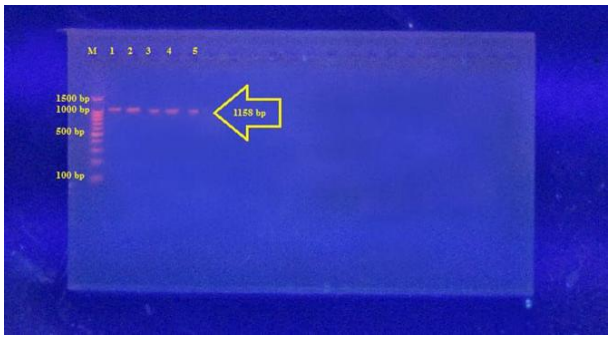

Figure 2. *SAG3* gene DNA Detection

T.gondii parasite (*SAG3* gene) PCR product Electrophoresis was conducted on an agarose gel at a voltage=60 for thirty minutes, employing the fluorescent red dye Ethidium Bromide. The specimens exhibited a band corresponding to 1158 base pairs of DNA isolated from patients with cancerous breast tissues.

TABLE 7. Relationship of toxoplasmosis with cancer kind

cancer kind	***T.gondii***				**Total**		X^2 **p-magnitude**	**OR (95 percent CI)**
	Ve+		**Ve-**					
	N	percent	N	percent	N	percent		
Invasive ductal carcinoma	16	94.1percent	126	94.7percent	142	94.7percent	0.011 0.915	0.89 (0.103-7.70)
Lobular ductal carcinoma	1	5.9percent	7	5.3percent	8	5.3percent		Ref.
Total	17	100percent	133	100percent	150	100percent		

The present investigation detected the highest infection rate with the parasite *T.gondii*, as shown in Table 2. The findings revealed that all kinds of breast carcinoma are susceptible to infection with *T.gondii*,

and there is no specific kind of infection. The findings detected that the kind most affected by females is invasive ductal carcinoma because it is the most popular kind of breast carcinoma.

TABLE 8. Relationship of *T.gondii* with breast carcinoma grade

cancer grade	***T.gondii***				**Total**		***X*² p-magnitude**	**OR (95 percent CI)**
	Ve+		**Ve-**					
	N	percent	N	percent	N	percent		
Grade 1	0	0.0 percent	9	6.8 percent	9	6.0percent	1.452 0.484	-
Grade2	9	52.9 percent	73	54.9 percent	82	54.7percent		Ref.
Grade3	8	47.1 percent	51	38.3 percent	59	39.3percent		0.79 (0.28-2.17)
Total	17	100 percent	133	100 percent	150	100percent		

The present investigation also detected the grades of breast carcinoma in *T.gondii* patients, and the statistical findings detected which grade of cancer is at risk for infection with *T.gondii*, as shown in Table (3).

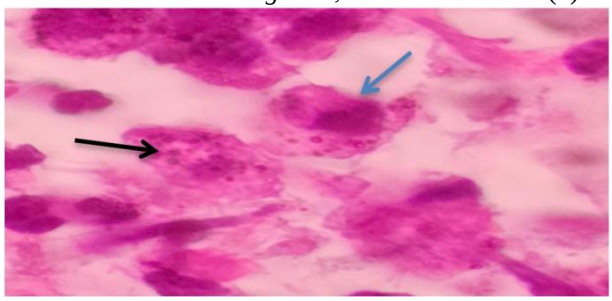

Figure 3. A tissue section from breast carcinoma is affected by the *T.gondii* parasite, the Cytoplasm of macrophage cell *s, and the T.gondii* parasite (1000x H&E).

The current research findings align with a survey conducted to ascertain the existence of the parasite in 900 patients in China, as reported by [28], [29], where the parasite's DNA was obtained from the cancer

tissues of 510 patients, and the *T. gondii* B1 gene was amplified utilizing a nested PCR technique. The survey investigation identified an overall infection incidence of 35.56 percent among patients of cancer categorized by organ kind [30]. Patients with lung cancer had the most remarkable prevalence rate of the *T. gondii* parasite at 60.94percent, then patients with cervical cancer at 50 percent, patients with brain cancer at 42.31 percent, and patients with endometrial cancer at 41.67percent. The infection of *T. gondii* in patients with cancer was significantly associated with soil exposure and the consumption of undercooked meat [31]. The infection rate in the breast tissues was reduced in the present study due to the potential non-uniform distribution of the parasite throughout the sampled tissues or biopsies. Several studies indicate that the hormonal system in tissues contaminated with the parasite *T.gondii* significantly contributes to the parasite's stability by affecting its tissue activities and enabling it to exploit host cells for its benefit. The parasite *T.gondii* can manipulate the immune system of infected individuals, utilizing hormonal alterations to evade immune detection by binding to specific receptors on the parasite, thereby inhibiting the action of host-secreted antibodies.

Furthermore, the immune reaction to *T.gondii* might contribute to neurological and histological alterations. *T.gondii* identified the uneven necrosis distribution in infected tissues, and there is definitive evidence of the parasite's presence in neurons. It induces infection by the *T.gondii* parasite [32]. In tissue, injury induces the synthesis of various inflammatory and cytokines mediators that facilitate inflammation in neighboring blood vessels, fibrous tissue, and immune cells, resulting in proliferation and hypertrophy of the tissues [33]. Proliferating epithelial cell layers and faulty cuboidal or basal cells in prostate tissue during the rapidly expanding phase of chronic toxoplasmosis inside host cells [34].

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Arabic Abstract

تم إجراء هذه الدراسة في نوفمبر 2023، وشملت جمع 150 عينة من أنسجة سرطان الثدي الخبيث المغطاة بشمع البارافين، والتي تم تشخيصها من قبل أخصائي، بالإضافة إلى 50 عينة من أنسجة أورام حميدة. تم الحصول على عينات مجموعة التحكم من مختبرات مدينة الإمام الحسين الطبية(IHMC) ، ومختبر مستشفى الكفيل التخصصي(KSHL) ، ومختبر السجاد التخصصي (SSL) لتحليل الأنسجة والخلايا وتحديد الأورام. شملت الدراسة مجموعتين: الأولى تضمنت 66 سيدة مصابة بسرطان الثدي ولم يتلقين العلاج الكيميائي، بينما تضمنت الثانية 84 سيدة تلقين العلاج الكيميائي. تم استخدام تقنية PCR على الحمض النووي المستخرج من أنسجة المرضى في جميع المجموعات، مع التركيز على تضخيم الجينات B1وSAG3لتحديد انتشار مرض التوكسوبلازما. أظهرت النتائج أن معدل الإصابة بالتوكسوبلازما بين مرضى سرطان الثدي بلغ 11.3%، مقارنة بـ 4% بين الأفراد الأصحاء. كما أشارت البيانات إلى أن معدل الإصابة كان أعلى بين السيدات اللواتي خضعن للعلاج الكيميائي، بسبب ضعف المناعة وزيادة خطر العدوى، مع زيادة بنسبة 3.7% في احتمالية إصابة المريضات بسرطان الثدي مقارنة بالمشاركات الأصحاء.