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ORIGINAL RESEARCH

Detection of *Toxoplasma gondii* and High-Risk Human Papillomaviruses in FFPE Malignant and Benign Breast Lesions Using Real-Time PCR

Selma Usluca[1,2], Ayfer Bakir[2], Ata Türker Arikok[3], Gizem Korkut[2], Gulsah Ceylan Yagiz[2], Murat Alper[3]

[1]Department of Medical Microbiology, Atilim University Medical School, Ankara, Türkiye; [2]Department of Medical Microbiology, University of Health Sciences Ankara Etlik City Hospital, Ankara, Türkiye; [3]Department of Pathology, University of Health Sciences Ankara Etlik City Hospital, Ankara, Türkiye

Correspondence: Selma Usluca, Department of Medical Microbiology, Atilim University Medical School, Kızılcasar District, 1184. Street, No: 13, 06830 Incek, Golbası, Ankara, Türkiye, Tel +90 5052537123, Email selmausluca@gmail.com

Objective: Breast cancer is the most prevalent malignancy among women. In recent years, it has been suggested that various pathogens such as *Toxoplasma gondii* (*T. gondii*) and human papillomavirus (HPV) may play a potential role in the development of breast cancer. This study aimed to determine the prevalence of *T. gondii* and HPV infections in formalin-fixed paraffin-embedded tissue samples of breast cancer patients using real-time PCR.

Methods: The study included 136 paraffin-embedded biopsy samples with w confirmed malignant breast tumor diagnosis and 50 breast tissue samples diagnosed as benign breast lesions, serving as controls. The presence of *T. gondii* DNA and high-, medium-, and low-risk HPV genotype DNAs were investigated using the real-time PCR method. First, deparaffinization was performed using xylene and alcohol, followed by DNA extraction and real-time PCR amplification.

Results: The most common histopathological types of malignant breast carcinoma were invasive carcinoma (n=82; 60.3%), invasive lobular carcinoma (n=26; 19.1%), invasive ductal carcinoma (n=8; 5.9%), and mixed invasive carcinoma (n=8; 5.9%). According to the Modified Bloom-Richardson classification, 55.15% of malignant breast tumor samples were grade 2, 32.4% were grade 3, and 12.5% were grade 1. Real-time PCR analysis did not detect *T. gondii* DNA or HPV DNA in any of these samples.

Conclusion: Our findings do not support a role of *T. gondii* and HPV in breast cancer development. To better understand the possible relationship between breast cancer and these pathogens, further studies with larger sample sizes, diverse diagnostic methods, and broder geographical coverage are necessary.

Keywords: breast, cancer, *Toxoplasma gondii*, high-risk human papillomaviruses, PCR

Introduction

Breast cancer is the most prevalent malignancy among women, occurring approximately 100 times more frequently in women than in men. Environmental factors such as genetic mutations, hormonal fluctuations, breastfeeding duration, obesity, smoking, and alcohol consumption play a crucial role in its development.[1]

According to GLOBOCAN 2022 estimates, approximately 2.30 million new cases of breast cancer were reported in women in 2022. In the same year, breast cancer ranked as leading cause of cancer-related death in women, causing approximately 670,000 deaths.[2]

In recent years, research on the involvement of infectious agents in breast cancer pathogenesis has intensified.[3,4] Studies suggest that both parasites and viruses may contribute to breast cancer development. Among parasites, *Toxoplasma gondii* (*T. gondii*) and among viruses, *mouse mammary tumor virus, simian virus 40, human papilloma virus* (HPV), *herpes simplex virus* (HSV), *cytomegalovirus* (CMV), *Epstein-Barr virus* (EBV) and *human herpes virus 8* (HHV-8) have been proposed as potential oncogenic triggers in breast cancer.[3,5,6]

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T. gondii is an obligate intracellular protozoan that infects nearly one-third of the global population. It has been reported to secrete antigens that inhibit tumor growth and exhibit anticancer properties in animal models.[7] Recent experimental research further supports this; in a mouse breast cancer model, *T. gondii* has been shown to induce antitumor effects by enhancing strong Th1-mediated immune responses, characterized by increased IFN-γ production and CD8+ T cell percentages.[8] However, recent studies have detected *T. gondii* DNA in malignant breast tissues, suggesting a potential link between toxoplasmosis and breast cancer.[3] Additionally, *T. gondii* has been associated with Hodgkin lymphoma, leukemia, melanoma, breast cancer, and multiple other malignancies.[9–12] Tissue cysts of *T. gondii* can be found in the brain, myocardium, eye, breast, and skeletal muscle, potentially elevating cancer risk. Latent toxoplasmosis may act as a tumor promoter in various malignancies.[13] Intracellular pathogens like *T. gondii* can compromise cellular barriers, leading to an increase in oncogenic mutations.[11,14,15] Furthermore, these pathogens can inhibit apoptosis by modulating gene expression through miRNAs, thereby making immunocompromised individuals more vulnerable to tumor progression.[11–13] It has also been suggested that *T. gondii* may disrupt the immune response by interfering with hormonal signaling.[10]

HPV is the most common sexually transmitted infection worldwide.[14] Although HPV is predominantly associated with cervical cancer, several molecular and epidemiological studies have reported the presence of HPV DNA in breast cancer tissues, suggesting a possible association with breast oncogenesis.[16–18] The integration of the HPV genome into the host DNA can cause chromosomal instability, thereby triggering carcinogenesis.[15]

Several theories have been proposed to explain HPV transmission to breast tissue, including hematogenous spread, direct epithelial transfer, or activation of latent virus due to immunosuppression.[19] The literature remains divided on whether HPV is a causal agent, a cofactor, or merely a bystander.[20]

A large-scale population-based cohort study from Taiwan reported that HPV infection may be associated with a significantly increased risk of breast cancer.[21] In contrast, a recent study from Northern Thailand found no significant association between HPV infection and breast cancer risk, suggesting that this relationship may vary by population and geographic region.[22] The role of *T. gondii* and HPV in breast cancer pathogenesis remains controversial. Further studies in this field could enhance our understanding of breast cancer etiology and contribute to the development of targeted therapeutic strategies.

The study aimed to determine the prevalence of *T. gondii* and HPV in formalin-fixed paraffin-embedded (FFPE) breast cancer tissue samples using the real-time PCR.

Materials and Methods

Study Population and Sample Selection

The study included archived FFPE breast tissue samples from women aged 18 years and older diagnosed with malignant breast cancer or benign fibrocystic breast disease. A total of 136 histologically confirmed malignant breast tumor samples and 50 benign breast lesion samples, archived between June 1, 2023 and February 29, 2024 were evaluated using molecular methods. All tissue sections were re-evaluated by board-certified pathologists to ensure the presence of sufficient tissue for molecular analysis.

Inclusion and Exclusion Criteria

Patients aged 18 years or older with a definitive histopathological diagnosis of malignant breast cancer or benign fibrocystic breast disease and sufficient FFPE biopsy material for molecular analysis were included in the study. Additionally, the first samples of patients with more than one sample were included in the study. The study excluded biopsies of metastatic lesions from outside the breast, samples diagnosed with conditions other than breast cancer or fibrocystic changes, tissue samples collected after chemotherapy, and cases that had insufficient tissue volume.

Sample Size Estimation and Power Analysis

In the planning stage of the study, we decided that we needed at least 100 samples (50 with cancer and 50 without) based on a power analysis that assumed a medium effect size (Cohen's d = 0.6), a significance level of 0.05, and a statistical

power of 80%. However, to make the study more representative, we ended up including 136 cancer cases and 50 non-cancer cases because we had enough archived FFPE tissue samples. A follow-up power analysis done with the final number of samples showed that the study had a statistical power of over 89% to find important differences between the groups. This result strengthens the reliability of the study's negative findings. All statistical tests were two-tailed, and a p-value of <0.05 was considered statistically significant.

Clinical and Histopathological Data Collection

Patients' demographic and clinical data, such as age, tumor location (right or left breast), histological grade (based on Modified Bloom-Richardson classification), and histopathological subtype, were obtained from the hospital's electronic medical record system. Figure 1 illustrates a representative breast biopsy section showing invasive carcinoma (Hematoxylin and Eosin, ×10 objective).

Tissue Sections and Processing

Strict precautions were taken to prevent cross-contamination during tissue preparation. Two 5 µ thick sections were cut from each FFPE tissue block using sterile, disposable blades and transferred into 1.5 mL screw-cap tubes. Samples were stored at room temperature until further processing.

Deparaffinization Protocol

For deparaffinization, 1000 µL of xylene (Merck Company, Germany) was added to each tube, incubated at 45°C for 15 min, and centrifuged at 10,000 rpm. The supernatant was discarded, fresh xylene was added again, and the sample was centrifuged once more. The pellet was washed twice with 98% ethanol to remove any residual xylene. Ethanol was then removed, and the pellet was allowed to evaporate completely before being collected for DNA extraction.[23]

DNA Extraction and Quantification

Before DNA extraction, pretreatment was performed using the Zybio EXM3000 automatic extraction device (Zybio Inc., China). DNA was then extracted using the Biospeedy® Rapid Nucleic Acid Extraction Kit (Bioeksen R&D Technologies Inc., Istanbul, Türkiye) following the manufacturer's instructions.

The obtained DNA concentration and purity were assessed using a Nano-Drop 2000 spectrophotometer (Thermo Scientific, USA) to determine its suitable for PCR amplification. For each polymerase chain reaction (PCR) experiment 100 ng DNA template was used. DNA extracts were stored at −80 °C until real-time PCR analysis.

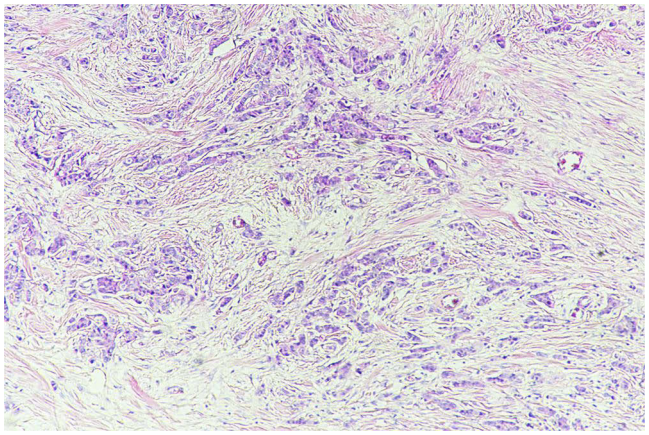

Figure 1 Breast biopsy section showing invasive carcinoma (Hematoxylin and Eosin, ×10 objective).

Real-Time PCR Procedure

Extracted genomic DNA was amplified using the Biospeedy *Toxoplasma gondii* RT-qPCR Kit (Bioeksen R&D Technologies Inc., Istanbul, Türkiye) which contains *T. gondii*-specific primers, and the Biospeedy *Human Papillomavirus* Genotyping qPCR Kit (Bioeksen R&D Technologies Inc., Istanbul, Türkiye), which contains primers specific to high-risk HPV types. The real-time PCR method was performed using the Magnetic Induction Cycler-PCR (Bio Molecular System, Australia) according to the manufacturer's recommendations.

The primers in the test kit were designed to target the Surface Antigen Gene (SAG3) region of *T. gondii* and the E1–E7 and L1 regions of HPV. The Human Papillomavirus Genotyping qPCR Kit was capable of detecting 28 different high-, intermediate-, and low- risk HPV types, including types 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, 68, 69, 70, 73, 82.

Contamination Control Measures

To prevent contamination during PCR analyses, the minimum precautions required for molecular studies were strictly applied. Sample processing, pre-amplification, and post-amplification procedures were conducted in separate areas using appropriate personal protective equipment. All equipment and surfaces were decontaminated before and after each procedure. Samples were stored separately from kits and reagents. To minimize contamination risk, reagents were prepared in aliquots, filtered pipette tips were used, and gloves were changed frequently during all procedures.

Interpretation of PCR Results

Positive and negative controls were included in the *T. gondii* and HPV analysis series. The quality of the extracted DNA was confirmed by demonstrating successful DNA extraction and simultaneous amplification of the human housekeeping gene in the absence of PCR inhibitors. Test results were evaluated based on amplification curves and cycle threshold (CT) values, following the manufacturer's recommendations.

Statistical Analysis

Statistical analyses were performed using SPSS 25 (IBM Corp., Armonk, NY, USA). In the study, descriptive data were presented as categorical variables (n, percentage [%]) and continuous variables as mean ± standard deviation (SD). The Kolmogorov–Smirnov test was used to assess the normality of continuous variables. Pearson's chi-square test was applied to compare categorical variables, while the independent sample *t*-test was used to compare quantitative parameters. A p-value of <0.05 was considered statistical significant in all analyses.

Ethical Approval

This study was conducted in accordance with the principles of the Declaration of Helsinki with the approval of the ethics committee of the hospital (approval number: AEŞH-BADEK-2024-208, approval date: March 6, 2024). As this was a retrospective study using archived and anonymized FFPE tissue samples without patient identifiers, the requirement for individual informed consent was waived by the ethics committee, in accordance with national and institutional ethical guidelines.

Results

The ages of the patients included in this study ranged from 23 to 89 years, with a mean age of 52.98±14.28 years. The mean age of malignant breast carcinoma cases (n=136; 73.1%) was 57.21±12.62 years (age range 30–89), while the mean age of patients with fibrocystic changes (n=50; 26.9%) was 41.48±12.08 years (range 23–74). The age difference between the two groups was statistically significant ($p<0.001$). Of the malignant breast tumor biopsy samples, 75 (55.1%) were obtained from the left breast, while in the benign breast lesion group, 29 (58%) of biopsies were obtained from the left breast.

The most common histopathological types of malignant breast carcinoma were invasive carcinoma (n=82; 60.3%), invasive lobular carcinoma (n=26; 19.1%), invasive ductal carcinoma (n=8; 5.9%) and mixed invasive carcinoma (n=8;

Table 1 Histopathological Distribution of Malignant Breast Biopsy Samples

Tumor Type	n	%
Invasive carcinoma	82	60.3
Invasive lobular carcinoma	26	19.1
Invasive ductal carcinoma	8	5.9
Mixed invasive carcinoma	8	5.9
Invasive mucinous carcinoma	7	5.1
Invasive tubular carcinoma	2	1.5
Invasive adenocarcinoma	1	0.7
Papillary carcinoma in situ	1	0.7
Ductal carcinoma in situ	1	0.7
Total	136	100

5.9%) (Table 1). Other histopathological types included invasive mucinous carcinoma, invasive lobular carcinoma, invasive adenocarcinoma, papillary carcinoma in situ, and ductal carcinoma in situ, which together accounted for 8.8% of malignant cases. According to the Modified Bloom-Richardson classification, 55.15% of malignant breast tumor samples were Grade 2, 32.35% were Grade 3, and 12.5% were Grade 1. Real-time PCR analysis did not detect *T. gondii* DNA or HPV DNA in the examined tissue samples.

Discussion

Although *T. gondii* has been proposed to possess oncogenic potential, epidemiological evidence supporting this hypothesis remains limited (El Skhawy, 2023). Factors such as immunosuppression and reactivation of latent infection are commonly discussed mechanisms in this context. Several studies have reported higher seroprevalence of *T. gondii* antibodies (IgG) in breast cancer patients compared to non-cancerous individuals, suggesting a possible link between toxoplasmosis and tumor development (Liu et al, 2019; Mahmood, 2019). This raises the possibility that either reactivation of chronic infection or increased susceptibility to new infection due to weakened host immunity may contribute to cancer pathogenesis. However, no direct causal relationship has been definitively proven.[14,15,24]

Several investigations have evaluated *T. gondii* presence using both serological and molecular approaches. Positivity rates for *T. gondii* DNA via PCR in blood samples of cancer patients vary widely across studies, likely due to differences in immune status, geographic location, and genetic background.[13,15,25]

Molecular detection of *T. gondii* in FFPE breast cancer tissue remains rare in the literature. The first study to identify *T. gondii* DNA in breast cancer biopsies was conducted by Kalantari et al,[24] reporting 3 positive samples among 29 malignant cases. However, two of those belonged to cancer patients and no statistically significant correlation was found. In our study, no *T. gondii* DNA was detected in either malignant or benign breast tissue samples.

Our negative findings may be related to technical limitations, such as DNA degradation in FFPE tissues, low pathogen load, or the presence of PCR inhibitors. These factors may have reduced the sensitivity of the molecular detection methods used. Similar negative results have been reported in other studies using FFPE materials,[24,26,27] indicating that sample type and quality are critical for accurate detection. For more reliable results, future studies should consider the use of fresh or frozen tissue samples, and apply more sensitive molecular techniques such as nested or multiplex PCR.[14,28] In addition, analyzing both FFPE and fresh samples from the same patients would help evaluate the impact of sample preservation on detection outcomes.[15]

HPV DNA detection in breast cancer tissues shows a wide range of positivity in the literature, with reported rates varying from 0% to 86%.[16–18,29–35]

Specimen type (fresh tissue vs FFPE), DNA integrity (DNA integrity may be compromised especially in FFPE tissues), viral load in the tissue sample and sensitivity of PCR methods used are the main methodological differences highlighted in these studies.[16,30–32,34] Diagnostic techniques such as PCR, in situ hybridization (ISH), and immunohistochemistry (IHC) also yield different results depending on their sensitivity and specificity.[28,36]

In this study, HPV DNA was not detected in malignant breast tumor tissues. In line with these findings, our results suggest that HPV may not have a significant role in the pathogenesis of breast cancer in our sample set. However, considering the limitations of using only archived FFPE tissues, this conclusion should be interpreted with caution.[29,37,38]

To better understand how HPV and *T. gondii* might be linked to breast cancer, future research should involve larger and more varied groups of people, use new tissue samples, and mix different scientific methods like molecular, serological, and histological techniques. Standardization in methodology will also be essential to ensure consistency and comparability across studies.[34]

This study has several limitations. The tissue samples were only taken from old FFPE (formalin-fixed, paraffin-embedded) materials, which might have damaged the DNA and lowered the quality of the samples, possibly affecting how reliable the molecular analyses are. The PCR method used can yield false-negative results in cases with low viral or parasitic load. The absence of positive cases precluded the application of more sophisticated statistical approaches, complicating the search for potential causal linkages.

Additionally, the retrospective and single-center nature of the study, as well as the relatively limited sample size, may restrict the generalizability of the findings. The lack of serological and immunohistochemical methods also hindered the confirmation of molecular findings through alternative techniques.

The contribution of this study to the literature is that it is one of the few studies reported from Turkey on this subject.

In conclusion, although neither *T. gondii* nor HPV DNA was detected in malignant or benign breast tissue samples in this study, these findings should be interpreted with caution due to the limitations mentioned above.

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Disclosure

The authors report no conflicts of interest in this work.

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