ORIGINAL ARTICLE

Human Mammary Tumor Virus Detection and APOBEC3G Expression in Breast Cancer: A Molecular Analysis of Iraqi FFPE Tissues

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ABSTRACT

Background*: Breast cancer remains a leading cause of morbidity and mortality globally, and emerging evidence implicates viral oncogenesis in its pathogenesis. Geographical variations in viral prevalence, such as Human Mammary Tumor Virus (HMTV) and host immune factors, such as APOBEC3G, highlight the need to explore their roles in carcinogenesis. Formalin-fixed paraffin-embedded (FFPE) tissues offer a valuable resource for retrospective molecular studies, although challenges, such as DNA degradation, persist.* ***Objective****: This study aimed to investigate the presence of HMTV and evaluate APOBEC3G expression in FFPE breast tissues from Iraqi women diagnosed with invasive ductal carcinoma (IDC) and benign fibroadenoma to assess their potential roles in breast cancer development.* ***Methodology****: A total of 100 FFPE samples (50 IDC and 50 fibroadenomas) were analyzed. DNA extraction, singleplex PCR (for the HMTV env gene), and gel electrophoresis were performed for viral detection. APOBEC3G expression was quantified by RT-qPCR, using the 2−ΔΔCt method.* ***Results****: HMTV sequences were detected in 22/50 IDC cases (44%) but were absent in controls. APOBEC3G expression was significantly upregulated in IDC (mean fold-change: 0.829 vs. 0.238 in controls; p = 0.015).* ***Conclusion****: HMTV is associated with IDC, supporting its potential role in breast carcinogenesis. Elevated APOBEC3G expression in patients suggests its involvement in tumor progression, although inconsistencies in significance warrant further investigation. These findings underscore the interplay between viral factors and host immunity in breast cancer, advocating expanded studies to validate biomarkers and therapeutic targets.*

INTRODUCTION

Breast cancer can present in less common forms such as triple-negative breast cancer, Paget's disease of the breast, and inflammatory breast cancer. Additionally, non-Hodgkin lymphoma and soft tissue sarcoma have been reported, albeit rarely, as manifestations of breast malignancies[1]. While the incidence of breast cancer in China has historically been low, recent data suggest a steady rise, with projections estimating that by 2022, over 100 per 100,000 Chinese women, particularly those aged 35–49, would be affected, totaling approximately 2.5 million cases. This underscores the importance of identifying and mitigating the risk factors for breast cancer [2].

In Iraq, several molecular studies have investigated the viral contributions to breast cancer, including the detection of Epstein-Barr Virus (EBV) in breast cancer tissues, emphasizing the relevance of exploring viral oncogenesis within the Iraqi population (Washil et al., 2025). Human Mammary Tumor Virus (HMTV) and other oncogenic viruses are also being increasingly studied owing to their varied prevalence and potential role in carcinogenesis [3,4].

Geographic variations in viral detection have also been reported. reported that MMTV sequences were present in 16% of breast cancer cases in Mexico and 12% in Iraq [5]. Similar variations in prevalence were also observed in studies from the United States, Italy, and Australia. A meta-analysis of 29 studies, encompassing 2,211 breast tissue samples from diverse regions, reported that 23% of patients with breast cancer tested positive for HPV DNA. HMTV prevalence varies widely across studies, ranging from 0% to 78%, whereas EBV DNA was detected in breast cancer tissues in 26 countries, with a prevalence ranging from 0% to 78.12% [6,7].

In Basrah and Iraq, poor survival outcomes have been associated with advanced stages and high-grade tumors. The 3-year survival rates for breast cancer patients were comparable to those of patients in other developing countries, but significantly lower than those

in developed nations. MMTV, a member of the genus *Betaretrovirus*, has been implicated in mammary tumors in both wild and laboratory mice [8].

Genetic predisposition also contributes to the risk of breast cancer. Inherited mutations in BRCA1 and BRCA2 significantly increase susceptibility, accounting for approximately 3–5% of breast cancers [9–11]. Interestingly, compared HMTV prevalence in women with BRCA-associated breast cancer to those with sporadic breast cancer, HMTV sequences were found in 30% of sporadic cases, but only 4% of hereditary BRCA-positive cases [12,13].

Among host restriction factors, Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like (APOBEC) proteins play a significant role in innate immunity and genomic stability. The APOBEC family includes 11 members: APOBEC1, AID, APOBEC2, APOBEC3 (A–H), and APOBEC4. Alternative splicing in APOBEC3B, APOBEC3H, and APOBEC3F contributes to their functional diversity. Although they share a conserved catalytic domain, APOBEC enzymes differ in their mutational targets, functions, and expression profiles across tissues [14]. This study aimed to investigate the presence of HMTV and evaluate APOBEC3G expression in FFPE breast tissues from Iraqi women with invasive ductal carcinoma (IDC) and benign fibroadenoma to assess its potential role in breast cancer development.

METHODOLOGY

A total of 10، formalin-fixed paraffin-embedded tissue (FFPE) samples, including 50 FFPE samples prepared from female patients clinically diagnosed with invasive ductal carcinoma (patient group) and 50 FFPE samples prepared from females clinically diagnosed with fibroadenoma (control group), were selected from the Histopathology Laboratory archive of Early Detection of Breast Cancer Clinic/ Al-Elawiya Maternity Teaching Hospital, which were processed during 2021, 2022, and 2023. The FFPE tissue was examined and diagnosed by histopathologists using hematoxylin- and eosin-stained histopathological examination as invasive ductal carcinoma and fibroadenoma, respectively.

From each FFPE block, 5–10 sections of 5 µm thickness were cut using a sterile microtome blade. The microtome was thoroughly cleaned with 70% ethanol to prevent cross-contamination between samples. The sections were then transferred into sterile nuclease-free microcentrifuge tubes. Deparaffinization was performed by adding 1 mL xylene, followed by two washes with 100% ethanol. The samples were then air dried. Materials Detection from each formalin-fixed paraffin-embedded (FFPE) tissue block, 5–10 sections of 5 µm thickness were cut using a sterile microtome blade. To prevent cross-contamination between the samples, the microtome was thoroughly cleaned with 70% ethanol after each use. Tissue sections were then transferred into sterile nuclease-free microcentrifuge tubes. Deparaffinization was performed by adding 1 mL xylene to each tube, followed by two washes with 100% ethanol. The samples were air dried at room temperature.

DNA was extracted from deparaffinized tissue sections, and its concentration and purity were assessed using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, USA). The A260/A280 absorbance ratio was used to evaluate DNA purity, with values between 1.8 and 2.0 considered acceptable for downstream PCR.

Singleplex PCR was conducted for the molecular detection of the HMTV APOBEC3G gene. The PCR reaction mixture (25 µL total volume) consisted of 12.5 µL of 2X GoTaq® Green Master Mix (Promega), 1 µL of each forward and reverse primer (10 pmol/µL), 2 µL of template DNA, and nuclease-free water to complete the volume. The thermal cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55–60°C for 30 s (optimized for each target gene), extension at 72°C for 45 s, and a final extension at 72°C for 5 min.

Primers specific for the env gene of HMTV were selected based on the sequences published previously [15], as shown in table (1)

Table 1: The singleplex PCR primers of *env* gene.

Primer Name	Primer sequence (5”-3”)	Product size (bp)	Reverence
ENV-F	GATGGTATGAAGCAGGATGG	250	[15]
ENV-R	CCTCTTTTCTCTATATCTATTAGCTGAGGTAATC		

The general properties of these primers were verified using OligoCalc oligonucleotide property calculator. The amplified PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide at a concentration of 0.5 µg/mL. A 100 bp DNA ladder was used as the molecular weight marker. Electrophoresis was performed at 100 volts for 45 minutes. DNA bands were visualized using a UV transilluminator and documented using a gel documentation system.

Detection of APOBEC3G Gene

APOBEC3G was detected in DNA samples extracted from formalin-fixed, paraffin-embedded (FFPE) tissue blocks obtained from two cohorts: female patients clinically diagnosed with invasive ductal carcinoma (study group) and females with fibroadenoma (control group). Primers targeting the human APOBEC3G gene (Reference Sequence: NM_021822.4) were designed using the Primer3 Input software (v0.4.0). The forward primer (AP3G-F: 5′-GGCTTTCTATGCAACCAGGCT-3′) and reverse primer (AP3G-R: 5′-CTCCAGGAGGTGAAGCAGGTA-3′) were validated for specificity and amplification efficiency using OligoCalc Oligonucleotide Properties Calculator, yielding a 140 bp amplicon. Singleplex PCR reactions were performed in a final volume of 25 µL containing 12 µL of 2X GoTaq® Green Master Mix (Promega, USA; including 10 mM dNTPs), 1 µL of each primer (20 µM), 4 µL of DNA template, and 7 µL of nuclease-free water. Thermal cycling was conducted under the following conditions: initial denaturation at 95°C for 3 min, 35 cycles of denaturation (96°C for 1 min), annealing (58°C for 30 sec), and extension (72°C for 2 min), followed by a final extension at 72°C for 8 min. The reactions were performed at 4°C after amplification.

The PCR products were resolved on a 1% agarose gel by electrophoresis at 100 V for 60 min. A 5 µL aliquot of each product was loaded with a DNA ladder (100–200 bp markers). Successful amplification of the APOBEC3G gene was confirmed by visualization of a 140 bp band under UV transillumination using a gel documentation system. This protocol was refined through iterative optimization to ensure specificity and efficiency, with the primer annealing temperature and reaction component volumes adjusted empirically. The observed amplicon size (140 bp) was aligned with the predicted product length to validate the accuracy of the assay. **Results:** The results of the present study showed that HMTV was remarkably associated with invasive ductal carcinoma, which was reported in 22 of 50 cases in the patient group.

The PCR result, measuring 140 bp, was obtained in a successful singleplex PCR experiment. Figure (2) shows that this gene was present during gel electrophoresis, where its band was found between the 100 bp and 200 bp bands of the 100 bp DNA ladder. This is considered to be an essential indicator of a successful response.

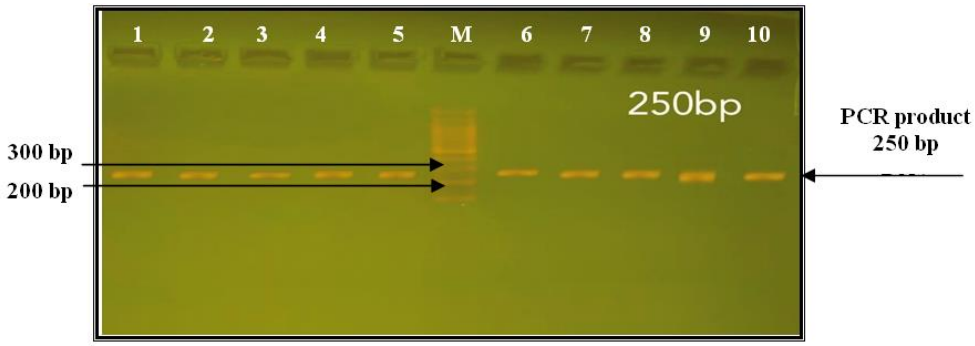

Fig. 1: Agarose gel electrophoresis of singlplex PCR product of *emv* gene of human mammary tumor virus utilizing 1% agarose gel at 100 volt for 1 hour. Lane M: 100 bp DNA ladder, lanes 1-10: Singlplex PCR product of *emv* gene

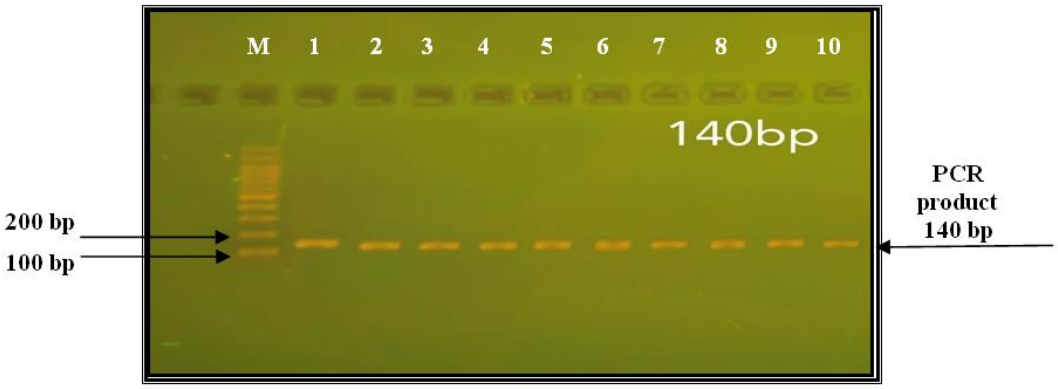

Fig. 2: Agarose gel electrophoresis of singlplex PCR products of *apobec3g* gene utilizing 1% agarose gel at 10volt/cm for 1 hour. Lane M: 100 bp DNA ladder, lanes 1-10: Singlplex PCR products of *apobec3g* gene.

The *apobec3g* gene expression of this study was detected using RT-PCR. The main purpose of this step was to measure the gene expression of the *apobec3c* and *gapdh* genes, and compare this expression between patients infected by HMTV (patient group) and those not infected with HMTV (control group) in order to evaluate the association between HMTV infection and *apobec3c* gene expression. The Ct values for gene amplification were recorded using quantitative RT-PCR software, as shown in figure (3).

APOBEC3G expression was analyzed using the Livak method (Table 1). Patients displayed higher ΔCt values (7.027) than the controls (8.169), with ΔΔCt values of 3.037 and 4.63, respectively. Consequently, patients exhibited a 3.48-fold higher expression (0.829) than controls (0.238). Table 2 confirms a statistically significant elevation in *APOBEC3G* expression in patients (0.829) compared to that in controls (0.238; p = 0.015).

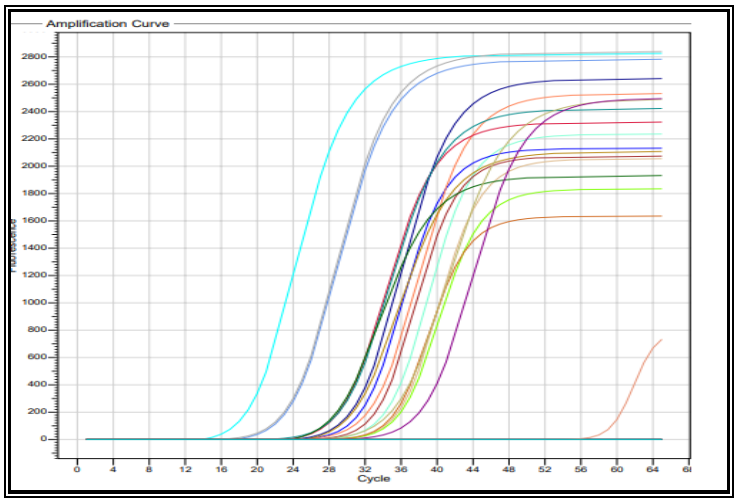

Fig. 3: Graph of *apobec3g* gene expression by RT-PCR.

Table 1: Comparative analysis of *APOBEC3G* gene expression using the livak equation (2^−ΔΔCt).

Group	*Means Ct of APOBEC3G*	*Means Ct of GAPDH*	*ΔCt (Means Ct of APOBEC3G)*	ΔΔCt	Fold of Gene Expression (2^−ΔΔCt)
Patient	37.05	30.02	7.027	3.037	0.829
Control	38.05	29.88	8.169	4.63	0.238

Table 2: Comparison of mean *APOBEC3G* expression between patient and control groups

Group	Mean ± SD	p-value
Patients	0.829	0.015*
Controls	0.238	

DISCUSSION

In the present study, singleplex PCR was utilized to detect HMTV in FFPE tissue samples using GoTaq® Green Master Mix and primers (ENV-F/ENV-R). This approach aligns with the study by Al-Alwany [13]conducted in Iraq, which reported the successful use of PCR to detect viral DNA in FFPE breast cancer tissues. The application of singleplex PCR for the detection of specific viruses, such as HMTV, is a widely accepted molecular diagnostic technique that enables precise and efficient amplification of targeted viral genes [16].

Furthermore, the 250 bp PCR product of the env gene obtained in the current study matched the expected size for the HMTV target gene amplified by the primers used. Similarly, Bevilacqua [14]detected a 250 bp PCR product of the env gene using specific primers for HMTV in breast cancer tissue samples[17]. Visualization of the PCR product by gel electrophoresis, with the band appearing between the 200 bp and 300 bp markers of a 100 bp DNA ladder, is a standard method for confirming PCR amplification. This analytical method is widely employed for viral DNA detection and is regarded as highly reliable. These findings are consistent with other local studies in Iraq that utilized gel electrophoresis to visualize PCR products and confirm the presence of viruses in breast cancer tissues[18].

The findings of the present study were consistent with the molecular techniques employed by Al-Mankhee *et al.*[19], who referred to the use of PCR and genome sequencing to analyze SARS-CoV-2 in Iraqi

patients, This highlights the diagnostic versatility of PCR in both infectious disease detection and tumor-related viral gene analysis. Additionally, the results are supported by Al-Janabi et al.[20], who employed PCR to detect adenovirus in children with gastroenteritis in Baghdad, reaffirming the effectiveness of PCR across different clinical samples.

While the detection of viral DNA in FFPE tissue samples using PCR is well documented, some studies have reported difficulties detecting low concentrations of viral DNA, especially due to DNA degradation and formalin-induced cross-linking in FFPE tissues. These challenges often result in poor-quality DNA, complicating viral detection [21,22]. In contrast, our successful detection of HMTV DNA indicates that our DNA extraction and preservation methods effectively minimized DNA degradation. However, some studies have observed variation in amplicon sizes, which may be due to differences in primer sequences or targeted regions. For example, a 300 bp PCR product has been reported in other studies targeting HMTV in breast cancer tissues, likely due to the use of alternative primers[8].

Singleplex PCR was employed to detect the APOBEC3G gene, and our findings are consistent with previous studies that have successfully used singleplex PCR to amplify APOBEC3G in various tissue samples [23,24], similar to the 140 bp fragment amplified in the current study. The use of FFPE tissue for genetic analysis has been extensively documented. Although we successfully amplified APOBEC3G from FFPE tissue, other studies have reported challenges due to DNA fragmentation and cross-linking caused by formalin fixation. Atanesyan et al.[25] noted that PCR amplification from FFPE samples often requires optimization of extraction protocols and PCR conditions. Furthermore, detecting low-expressed genes in FFPE samples can be difficult compared to fresh or frozen tissues due to RNA degradation and lower DNA quality, which may vary depending on tissue preservation methods and genetic stability[23].

Our study found no significant difference in APOBEC3G gene expression between the patient and control groups ($p = 0.440$), consistent with previous reports. Other local Iraqi studies similarly found no significant differences in APOBEC3G expression levels between malignant and benign breast tumors, suggesting that APOBEC3G may not serve as a reliable biomarker to distinguish these conditions in certain populations [26,27].

Conversely, the results of this study contrast with findings from other investigations that reported higher APOBEC3G gene expression levels in malignant breast tumors. Roelofs et al.[28] found significantly increased APOBEC3G expression in malignant breast cancer tissues compared to benign tumors, suggesting a potential role for the gene in more aggressive tumor types. Additionally, some studies conducted in Iraq have reported that APOBEC3G expression was significantly elevated in high-grade breast cancer tumors, implying that tumor grade and malignancy status may influence APOBEC3G gene regulation[29]. Therefore, studies with larger cohorts encompassing a wider range of tumor grades and molecular subtypes may produce different findings.

The APOBEC3G expression results in the current study showed no significant difference between patient and control groups, consistent with certain prior research, particularly within the Iraqi population. However, conflicting evidence from both local and international studies underscores the possible impact of tumor heterogeneity, methodological variations, and patient demographics on APOBEC3G gene expression patterns.

CONCLUSION

This study demonstrated the presence of HMTV DNA and differential expression of APOBEC3G in breast cancer FFPE tissues. These findings suggest that both viral factors and host gene regulation contribute to breast cancer pathogenesis. Further research involving larger sample sizes and functional assays is needed to clarify the precise mechanisms involved and to explore the potential clinical implications of these interactions.

Competing interests: The author declare that they have no competing interests
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Ethical approval: Ethical approval for the study was obtained from the Scientific Committee of the Biotechnology Department at the Institute of Genetic Engineering and Biotechnology, as well as the Al-Alawiya Hospital for Obstetrics and Gynecology, Iraq. The research was authorized formally by the Ministry of Health through the Baghdad Health Department, as documented in official letter No. 92155 which dated on June 13, 2023.
Availability of data and material: The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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