RESEARCH ARTICLE

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Immunohistochemical Detection of the Expressed *BRCA1* and *BRCA2* Proteins in Microenvironment of Malignant Breast Cancerous Tissues Infected with Human Mammary Tumor Virus

Saad Hasan Mohammed Ali[1]*, Khalil Ismail Abid Mohammed[1], Wifaq M. Ali[1], Suha A. AL-Fakhar[1], Shakir H. Mohammed Al-Alwany[2], Jinan M. Mousa[1]

Abstract

Objective: The objective of the study was to examine the prevalence of HMTV infection, its associations with breast malignant tissues, and the expression of *BRCA1* and *BRCA2* proteins. **Methods:** One hundred archival breast tissues, 40 biopsies from female patients with breast cancer (BC), and 20 healthy breast tissues from the control group were used in the study. Immunohistochemical analysis was conducted to detect the expressed *BRCA1* and *BRCA2* proteins. Digoxigenin-labeled HMTV probes were used in chromogenic in situ hybridization for the identification of HMTV in breast tumor tissues. The complementary sequence sites of the HMTV probe sequences were stained by NBT/BCIP as blue signals. **Results:** There were 12 out of 40 (30%) benign breast tumorous tissues and 14 out of 40 (35%) BC tissues, while healthy control breast tissues were 10% (2 out of 20 tissues). Positive immunohistochemical (IHC) reactions for *BRCA2* protein were observed in 12 out of 40 BC tissues (30.0%), 25% of benign breast tumorous tissues, and 5% of the control group. A significant ($p < 0.05$) statistical difference in the percentages of HMTV in the studied groups was found. **Conclusion:** HMTV might contribute to the development of subsets of benign and malignant breast tumors. The observed rates of defective or mutated *BRCA1* and *BRCA2* genes in healthy tissues indicate a role in the development of breast tumors.

Keywords: *BRCA1* gene- *BRCA2* gene- HMTV- IHC- CISH

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Introduction

Breast cancer (BC) is the most common and lethal malignancy in women. According to Globocan 2018 data, more than 2 million (11.6%) people worldwide are newly diagnosed with breast cancer. It also has the fourth-highest mortality rate (Sung et al., 2021). In addition to many biological characteristics of BC, earlier research by Claus et al., (1996) and Chen et al., (1999) revealed that 5–10% of unselected BC cases were diagnosed as hereditary breast cancers. These patients had a clear germ-line genetic mutation, and many of these genes were linked to breast cancers with a medium or low penetrance. Additionally, in recent years, more claims have been made regarding the inheritance of genetic changes contributing to the development of BC (Lima et al., 2019).

BRCA1 and *BRCA2* mutations (*BRCA1/2*) are significantly recognized to have both the strongest correlation and increase the lifetime risk of breast cancer, as well as ovarian, prostate, pancreatic, colorectal tumorigenesis, and/or carcinogenesis. This observation was made according to the results of 180,000 epidemiologically studied women and among a set of 8 recently identified genes that are mainly implicated in hereditary BC. *BRCA1* and *BRCA2* mutations (*BRCA1/2*) are important for DNA repair (Umarane et al., 2023), protection of the replication fork, regulation of both cell cycles, and gene transcription (Kuchenbaecker et al., 2017; Manchana et al., 2019). The large increase in the incidence of breast and ovarian cancers has been linked to the mutation or loss of the *BRCA1* gene, and the mutated *BRCA1/2* genes both have high penetrance susceptibility and a high link to most inherited breast cancers. The etiology of breast cancer was reported to be highly influenced by environmental variables (Laraqui et al., 2013). The mouse mammary tumor virus (MMTV), a type B retrovirus, has been identified as the primary cause of mouse mammary cancers. By inserting a mutagen or activating the transcription of oncogenes, the MMTV generated both premalignant lesions and malignant tumors

*[1]Clinical Communicable Diseases Research Unit, College of Medicine, University of Baghdad, Iraq. [2]College of Science, University of Babylon, Babylon, Iraq. *For Correspondence: saadhasanmohammedali@comed.uobaghdad.edu.iq*

in the mammary tissue (Parisi et al., 2022). Scientists have successfully amplified MMTV-like sequences from human breast cancers since 1995, and lately in breast tissues before the development of breast cancer, it was named human mammary tumor virus (HMTV), indicating a possible causal role in human breast carcinogenesis (San et al., 2017).

The present study was conducted to determine the prevalence of human mammary tumor virus (HMTV) infection and gene mutations in benign breast tumor tissues and primary invasive breast cancer tissues collected from a group of Iraqi patients.

Materials and Methods

Human subjects

The investigation was a retrospective case-control study, in which a total of 100 archival tissues from females with breast tumors were obtained. Forty (40) tissue tumor blocks from female patients with BC, of which 28 ductal BC, 7 medullary BC, and 5 lobular BC were dispersed among the malignant breast tumor group. In addition, 40 tissue blocks from female patients with benign breasts were enrolled as the patient control group, while an additional 20 healthy breast tissues from normal individuals were included as the healthy control group. The accompanying histological reports were the main basis for the initial diagnosis of the included archival tissue and a consultant pathologist who re-examined each case further confirmed the diagnosis.

Detection of human mammary tumor virus

For the detection of HMTV, a recent version of the chromogenic in situ hybridization (CISH) kit (Zyto Vision Gmb H. Fischkai, Bremerhaven, Germany) was used following the manufacturer's instructions by using specific digoxigenin-labeled oligonucleotide probes specified to target the HMTV DNA sequence achieved in the examined tissues. The main steps of CISH included deparaffinization, rehydration, permeabilization of the tissues, probe application, and chromogenic chain system application. The CISH signals were classified into positive and negative results. Also, CISH signal quantification was evaluated under light microscope, where the counting of CISH-positive cells was performed at 400x. The counting of 100 cells in ten different high–power fields was determined. Chromogenic in situ hybridization was given an intensity and a percentage score based on the strength of the positive signals and the number of signals, respectively (Baltzell et al., 2012).

Immunohistochemical detection of BRCA1 and BRCA2 proteins

The immunohistochemical (IHC) detection system (Abcam, England) was used to assay the *BRCA1* and *BRCA2* proteins by using primary antibodies specialized for the expressed protein products of the defective (or mutated) *BRCA1* and *BRCA2* genes.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS; version 26) program to evaluate the statistical significance of different relationships obtained in this study. A significant difference was considered as $p < 0.05$. The Chi-square test was used to detect the statistical significance between the variables in the current study.

Results

Age of patients with breast tumor group

The archival breast tissues were obtained from women with breast tumors who were 20 to 85 years old. The mean age of the breast cancer patients was 43.53+11.73 years, which was higher than the mean ages of the groups that served as controls; benign tumors (41.63+ 10.99 years) and healthy individuals (38.34+12.18 years). There were no statistically significant age differences ($p < 0.05$) between the groups under study (Table 1).

Age stratification of the studied breast tumor patients according to their histopathological diagnosis

The most commonly affected age stratum in the malignant breast tumor group was 41-60 years, constituting 55%. This was followed by people between the ages of 20 and 40, who made up 30% of the group, and people

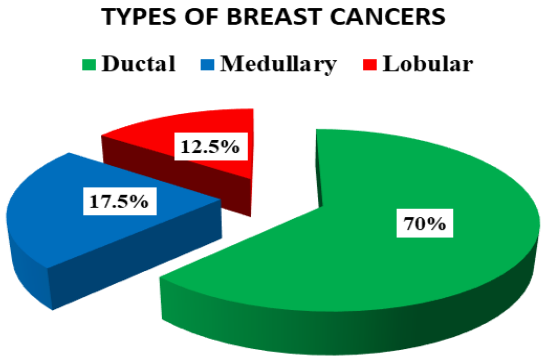

Figure 1. Distribution of Malignant Breast Cancer Groups According to Their Histological Typing

Table 1. Distribution of Breast Cancer Patients According to the Age

Studied Groups	N	Mean Age (Year)	SD	SE	Range		ANOVA test (P-value)
					Min.	Max.	
Control Healthy Individuals Group	20	38.34	12.18	3.73	30	72	
Benign Breast Tumors	40	41.63	10.99	2.92	20	55	P=0.07 non-Sig. ($p < 0.05$)
Breast Cancers	40	43.53	11.73	2.63	22	83	
Total	100						

SD, Standard Deviation; SE, Standard Error

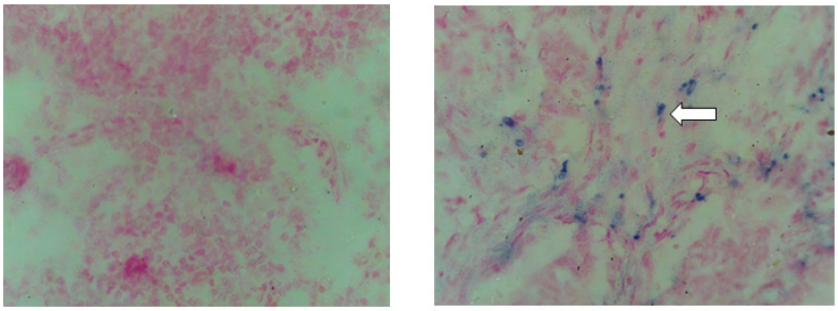

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Figure 2. The Light Microscopic Demonstration of HMTV- CISH Signals in Breast Tumor Tissues. Using Digoxigenin-Labeled HMTV probes that were stained by NBT/ BCIP (Blue) and counterstained by Nuclear Red Solution (Red) where blue signals were detected at complementarity sequences sites of probe sequences (arrowed). A- Negative HMTV–CISH reaction (40x). B- Positive moderate score and strong signal intensity HMTV–CISH reactions (40x).

Table 2. Analysis of Age Strata Distribution According to the Histopathological Diagnosis of Studied Cases

Age Strata / Year		Studied Group			Chi-Square
		Control Group	Benign Tumors	Breast Cancers	(p-value)
20 - 40	N	5	16	12	
	%	25	40	30	p = 0.04 (Sign.)
41 - 60	N	8	19	22	(p < 0.05)
	%	45	47.5	55	
61 - 83	N	7	5	6	
	%	35	12.5	15	
Total	N	20	40	40	
	%	100	100	100	
Chi-Square		A	B	C	
(p-value)		p = 0.04 sign. (p < 0.05)	p=0. 04 (Sign.) (p > 0.05)	p =0.03 (Sign.) (p < 0.05)	

between the ages of 61 and 80, who made up 15%. The age group with the highest prevalence of benign breast tumors was those between the ages of 41 and 60, accounting for 47.5% of cases, followed by those between the ages of 20 and 40 and those between 61 and 80, respectively, with 40% and 12.5% of cases. Highly statistically significant differences ($p < 0.05$) were observed among the age strata groups, as displayed in Table 2.

Histopathological typing of the studied breast cancer groups

The ductal BC (28 cases; 70%) was the most prevalent kind of breast cancer in the study, followed by the medullary BC (7 cases; 17.5%) and the lobular BC (5 cases; 12.5%). Highly statistically significant differences ($p < 0.05$) were

Table 3. Tumor Grading of the Studied Breast Cancers

Breast Cancer	N	%	Chi-Square
Grade / Differentiation			(P-value)
Well	10	25	p = 0.232 (Non-Sign.)
Moderate	14	35	(p > 0.05)
Poor	16	40	
Total	40	100	

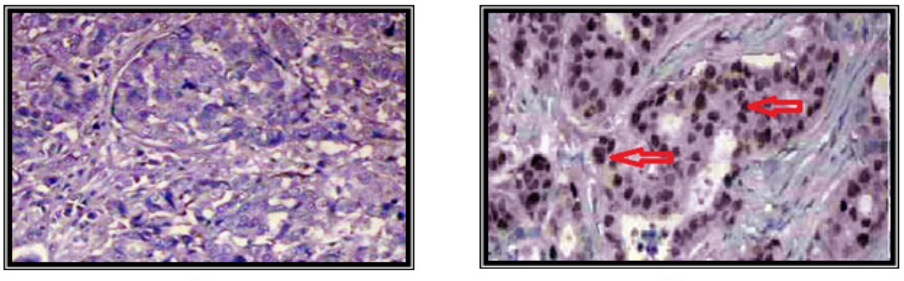

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Figure 3. Over-Expressed *BRCA1* Protein on Microscopic Examination of the Studied Breast Tumor Tissues: chromogenic DAB- Stained (brown) and Mayer's hematoxyline-counter stained (blue). Breast tumor tissues showing negative BRCA1 protein –IHC reaction (40x). Positive breast tumor tissues for BRCA1 protein –IHC reaction showing the low score and weak signal intensity (40X).

Table 4. Distribution of HMTV-CISH Signals among Female Patients with Breast Tumors

HMTV-CISH Signal		Studied Patients			Chi-Square (p-value)
		Healthy Control Group	Benign Tumors Group	Breast Cancers Group	
Positive	N	0	8	15	
	%	0	20	37.5	p=0.03 (Sign.)
Negative	N	20	32	25	(p < 0.05)
	%	100	80	62.5	
Total	N	20	40	40	
	%	100	100	100	
Chi-Square		A	B	C	
(p-value)		p = 0.123	p = 0.04	p = 0.04	
		Non-Sign.	Sign.	Sign.	
		(p > 0.05)	(p < 0.05)	(p > 0.05)	

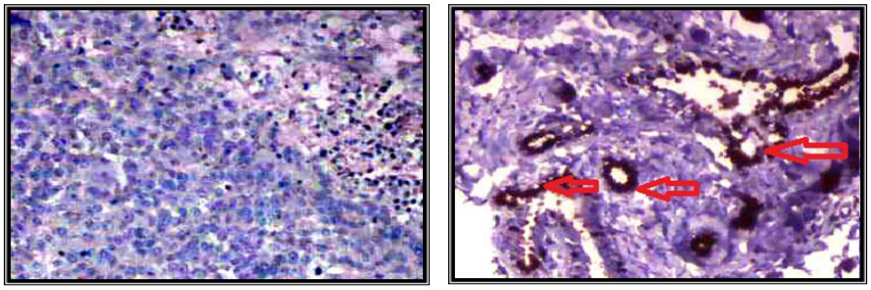

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Figure 4. Light Microscopic Appearance of Overexpressed *BRCA2* Protein in Breast Tumorous Tissues; Stained by chromogenic DAB (brown) and counter-stained with Mayer's Hematoxylin (blue). Negative BRCA 2–IHC reaction (40X). B. Positive BRCA 2–IHC reaction showing moderate score and high signal intensity (40X)

observed among histopathological types of BC.

Histopathological grading of breast cancers

Table 3 displays the distribution of breast cancers according to the Scarf-Bloom-Richardson grading system used in BC. The results revealed that poorly differentiated breast cancers accounted for 40% (16 of 40 cases), moderately and well-differentiated grades for 35% (14 out of 40 cases), and 25% (10 out of 40 cases), respectively. The analysis of breast cancers according to their grading

Table 5. Distribution of Immunohistochemical Detection Results of *BRCA1* Protein in Breast Tumor Tissues According to the Frequency of Signal Scoring

	IHC Reaction Results of *BRCA1* Protein		Studied groups			Chi-Square (p-value)
			A.H. Breast Control Tissues	Benign Breast Tumor Tissues	Breast Cancer Tissues	
BRCA 1 Protein -IHC Scoring	Negative	N	18	28	26	
		%	90	70	65	p = 0.431
	Low	N	2	6	8	Non-sign.
		%	10	15	20	(p > 0.05)
	Moderate	N	0	4	5	
		%	0	10	12.5	
	High	N	0	2	1	
		%	0	5	2.5	
	Total	N	20	40	40	
		%	100	100	100	
	Chi-Square		A	B	C	
	(p-value)		p = 0.129	p = 0.355	P = 0.644	
			Non-sign.	Non-sign.	Non-sign.	
			(p > 0.05)	(p > 0.05)	(p > 0.05)	

Table 6. Frequency of Immunohistochemistry Results of *BRCA 2* Protein According to the Signal Scoring Grades

	IHC Reaction Results of *BRCA2* Protein		Studied groups			Chi-Square (p-value)
			Control Tissues	Benign Tumor Tissues	Breast Cancer Tissues	
Positive IHC- Scoring Results	Negative	N	19	30	28	p = 0.064
		%	95	75	70	Non-sign.
	Low	N	1	5	8	(p > 0.05)
		%	5	12.5	20	
	Moderate	N	0	4	3	
		%	0	10	7.5	
	High	N	0	1	1	
		%	0	2.5	2.5	
	Total	N	20	40	40	
		%	100	100	100	
	Chi-Square		A	B	C	
	(p-value)		p = 0.081	p = 0.392	p = 0.079	
			Non-sign.	Non-sign.	Non-sign.	
			(p > 0.05)	(p > 0.05)	(p > 0.05)	

Table 7. Spearman's rho Statistical Test for the Evaluation of the Studied Molecular Markers in Female Patients with BC in Relation to Results of HMTV Infections

Female Patients with Breast Cancers					
Spearman's rho Correlation		HMTV	*BRCA1*	*BRCA2*	Age
HMTV	r			0. 586	0.695
	p-value			0.006	0.06
BRCA 1	R	0. 443			
			r = 0.398		
			p = 0.044		
	p-value	0.003			
BRCA 2	R				
	p-value				
Type	R	0.348			
	p-value	0.04			
Grade	R	0.449	0.255	0.344	
	p-value	0.039	0.03	0.04	

showed a significant statistical difference ($p < 0.05$) between the poorly and well-differentiated grades of BC, and a non-significant difference was observed between poorly and moderately differentiated grades of BC.

CISH for HMTV in female patients with breast tumors

As observed in Figure 1, only 37.5% (15 out of 40 cases) in the BC group showed positive signals of CISH for the detection of HMTV, while the remaining 62.5% (25 out of 40 cases) revealed negative signals. Concerning the benign breast tumor group, 20% (8 out of 40 cases) showed positive CISH signals for HMTV detection, while the percentage of negative results for this virus in the benign breast tumors group was 80% (32 out of 40 cases). There was a significant ($p < 0.05$) difference in the percentages of HMTV among the studied groups as highlighted in Table 4.

Expression of BRCA1 protein in breast tumor tissues

In the malignant breast tumor tissues, 14 out of 40 (35%) showed positive signals for *BRCA1*-IHC detection. This includes 8 tissues (20%) with a low score (score I), followed by 12.5% (5 tissues), and 2.5% (1 tissue), respectively, with a moderate (score II) and high score (score III) as depicted in Table 5. The majority of benign breast tumor tissues (15%), represented by 6 out of 12 tissues, scored with a low signal, whereas 10% (4 out of 14 tissues) and 5% (2 out of 14 tissues), respectively, scored with a moderate score (score II) and a high score (score III). In the control tissues group, 10% (2 out of 2 tissues) have a low score (score I) as indicated in Figure 2. There were no statistically significant ($p > 0.05$) differences in the tissue scores.

Expression of BRCA2 protein in breast tumor tissues

Over-expressed *BRCA2* protein was detected by IHC in 30% (12 out of 40) breast cancer tissues and i5% (26 out of 40) benign breast tumor tissues. Meanwhile, only one tissue (5%), from the control tissues group, exhibited this overexpression (Figure 3). A high percentage (8 out of 12 tissues) included malignant breast tumor tissue, of which 7.5% (3 out of 12 tissues) and 2.5% (1 out of 12 tissues) have moderate and high scores, respectively. In the benign breast tumor tissues group, 12.5 % (5 out of 10 tissues) were observed to have low score (score I) while 10% (4 out of 10 tissues) and 2.5% (1 out of 10 tissues) had moderate and high scores, respectively. In the control tissue group, 5% (1 tissue) have a low score (score I). There were no statistically significant differences between these scoring grades ($p > 0.05$), as presented in Table 6 and Figure 4.

Correlations among the studied markers

The correlations among the studied markers, which included HMTV, *BRCA1*, *BRCA2*, age of female patients with breast cancers, grade, and type of breast cancers in female breast cancer patients are shown in Table 7. In

female patients with breast cancer, there was a strong positive relationship and highly significant correlation between HMTV and *BRCA1* as well as *BRCA2* ($r = 0.443$, $p = 0.003$ and $r = 0.58$, $p = 0.006$, respectively; $p < 0.01$). Also, a significant correlation, as well as a strong positive relationship, was observed between *BRCA1* and BRA2 in female patients with breast cancers ($r = 0.398$, $p = 0.044$; $p < 0.05$). In addition, there was a positive relationship between HMTV and grade, as well as the type of breast cancer ($r = 0.449$, $p = 0.03$ and $r = 0.348$, $p = 0.04$, respectively; $p < 0.05$). However, no significant relationships between HMTV and the age of female breast cancer patients were observed ($r = 0.695$, $p = 0.06$; $p < 0.05$). Moreso, there was a significant correlation between the grade of breast cancer tissues and both *BRCA1* and *BRCA2* ($r = 0.255$, $p = 0.03$ and $r = 0.344$, $p = 0.04$, respectively; $p < 0.05$).

Discussion

In the past, studies have revealed a significant association of HMTV infection with the presence of hormone receptors in the hormone–dependent tissues in the body and the development of cancer in those hormone-influenced tissues. The HMTV has been identified in 14-74% of human breast cancers, 16% of ovarian cancers, 36% of prostate cancers, and 10% of endometrial malignancies (Johal et al., 2010). In this regard, a previous study in Iraq by Ali et al., (2015) on HMTV infection in endometrial and uterine cervical cancers revealed HMTV in 16.7% of endometrial cancers and 13% of malignant uterine cervical tumors. This research was carried out in Iraq to examine the correlation between the rate of HMTV infections with the expressed proteins of mutated *BRCA1* and *BRCA2* in a group of Iraqi patients with breast cancers and benign breast tumors. The HMTV infections were analyzed using digoxigenin-labeled probes in a chromogenic in situ hybridization analysis, while the breast tumors were examined with a specialized immuno-enzymatic antigen detection system specific for defective and/or mutant BRCA1 and *BRCA2* proteins. In this study, 75% of the breast cancer samples were histopathologically evaluated as having moderate to poor differentiation. The histological grading is a critical factor in the risk assessment of patients with BC. In this respect, it was discovered that younger women with higher grade and proliferation tumors have more vascular invasive tumors compared to their older women counterparts. Also, it was reported that grade I BC patients had 10-year survival rates of around 80%, falling to grade III BC patients' rates of only 45% (Harvey and Everett, 2004).

In the group of breast cancers currently under study, 37.5% of all screened BC tissues showed positive CISH signal results for HMTV-DNA, while 20% of all screened benign breast tumorous tissues were also found to contain positive CISH signal results for HMTV-DNA. However, none of the control breast tissues did. A statistical analysis of the percentages of HMTV in the various groups of breast tissue under study revealed a significant difference ($p < 0.05$). The values obtained in the current investigation are higher than those from a study conducted in Mexico when BC samples from Mexican women with breast cancer showed a prevalence of MMTV-like sequences of 4.2% (Zapata-Benavides et al., 2007). Additionally, while HMTV sequences were found in the milk cells of 20.6% of the biopsy sample, HMTV-env was only detected in 7.6% of the DNA from a reference group of women. The findings of the present study corroborate other researchers' findings and hypotheses that HMTV was associated with an elevated risk for BC (Nartey et al., 2014). Higher HPV, EBV, and MMTV positivity was discovered to correlate and/or associate with the expression of estrogen and human epidermal growth factor receptor 2 receptors, as well as with the metastasis to lymph nodes and aggressiveness of BC in human patients who presented with higher-grade breast cancers (Naushad et al., 2017). However, neither the clinicopathological manifestations of BC illness nor the co-presence of HMTV with HPV and EBV was examined in these reports.

In human patients who presented with increased-grade breast cancers, higher positivity of HPV, EBV, and MMTV were observed to correlate and/or associate with the expression of receptors for both estrogen and human epidermal growth factor receptor 2, as well as with the metastasis to lymph node and aggressiveness of BC (Naushad et al., 2017). However, neither the clinicopathological manifestations of BC illness nor the co-presence of HMTV with HPV and EBV was examined in these reports. Studies from the Arab World have discovered limitations in the link between the clinicopathological features of BC disease and the co-occurrence of HPV, EBV, and HMTV in BC patients (El-Shinawi et al., 2016). Previous studies revealed that *BRCA1* and *BRCA2* accounted for 0.7–29% and 1.5–25% of mutations observed in hereditary breast and ovarian cancer syndrome (HBOC), respectively (Ramus and Gayther 2009). According to other research, 3–8% of breast cancer cases in the general population have a *BRCA1/2* mutation. However, 5–10% of breast cancers and 15% of ovarian cancers contain *BRCA1/2* gene abnormalities, which are both characterized by HBOC (Tikhomirova et al., 2005). EBV LMP-1 and EBNA-1 IHC reactions were detected in 32.4 and 35.3%, respectively, of breast cancer tissues from a group of female patients in an Iraqi study (Ali et al., 2017). Meanwhile, *BRCA1* and *BRCA2* proteins were detected in 47.1 and 41.2%, respectively, in BC tissues.

Since the majority of breast cancers are hereditary with racial and geographic variations, identifying the women at risk for BRCA-related HBOC syndrome and estimating *BRCA1/2* gene mutation and/or variations in patients with a family history appears to be a crucial task in the majority of developed nations (De Leeneer et al., 2009). According to a recent study, the risk of BC in older women depends on the type of pathogenic variants. Missense mutation variations, particularly those in the *BRCA1* gene, had a lower risk of BC than pathogenic truncated mutations (Li et al., 2022). It was discovered that by the age of 80, women with pathogenic variants (PV) of *BRCA1* PV and *BRCA2* PV, respectively, had cumulative incidences of BC of 72–80% and 69%. These findings were correlated

with different clinical, biological, and pathological characteristics (Kuchenbaecker et al., 2017).

In conclusion, the subset of breast cancer might have developed due to the significant detection rate of HMTV. Also, there was a correlation between the grades of the investigated breast malignant tissues and the expression of the *BRCA1* and *BRCA2* genes, as well as HMTV in BC. Patients may also shed light on their occurrence and contribution as an early event in the development of breast cancer. This in turn supports an etiologic hypothesis for HMTV with mutated and/or defective *BRCA1* and *BRCA2* genes in breast carcinogenesis. To identify where such infections were initially acquired and determine whether these HMTV infections are a source for a later malignant state in other body sites, it is therefore, recommended to evaluate HMTV prevalence in the population, particularly those with breast cancer as well as in other body tumors and cancers.

Author Contribution Statement

All authors participated in the conceptualization and design of the study, data collection, data analysis or interpretation, and preparation of the manuscript.

Acknowledgements

Data availability

The data will be available upon the request to the corresponding author.

Conflict of Interest

The authors declare that there is no conflict of interest.

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