

# Prevalence of MMTV-Like env Sequences and Its Association with BRCA1/2 Genes Mutations Among Egyptian Breast Cancer Patients

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Samah A Loutfy<sup>1</sup>  
Zeinab F Abdallah<sup>1</sup>  
Mohamed Shaalan<sup>2</sup>  
Mohamed Moneer<sup>3</sup>  
Adel Karam<sup>3</sup>  
Manar M Moneer<sup>4</sup>  
Ibrahim M Sayed<sup>5,6</sup>  
Amer Ali Abd El-Hafeez<sup>7,8</sup>  
Pradipta Ghosh<sup>8-10</sup>  
Abdel-Rahman N Zekri<sup>1</sup>

<sup>1</sup>Virology and Immunology Unit, Cancer Biology Department, National Cancer Institute, Cairo University, Cairo, Egypt; <sup>2</sup>Surgical Oncology Department, National Cancer Institute, Cairo University, Cairo, Egypt; <sup>3</sup>Surgical Oncology Department, Materia Teaching Hospital, Cairo, Egypt; <sup>4</sup>BioStatistics and Epidemiology Department, National Cancer Institute, Cairo University, Cairo, Egypt; <sup>5</sup>Department of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University, Assiut, Egypt; <sup>6</sup>Department of Pathology, School of Medicine, University of California, San Diego, La Jolla, CA, USA; <sup>7</sup>Pharmacology and Experimental Oncology Unit, Cancer Biology Department, National Cancer Institute, Cairo University, Cairo, Egypt; <sup>8</sup>Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA, 92093, USA; <sup>9</sup>Department of Medicine, University of California, San Diego, La Jolla, CA, 92093, USA; <sup>10</sup>Moore's Cancer Center, University of California, San Diego, La Jolla, CA, 92093, USA

Correspondence: Samah A Loutfy  
Virology and Immunology Unit, Cancer Biology Department, National Cancer Institute, Fom El-Khalig, Cairo, 11796, Egypt  
Tel +201 222840964  
Email samaly183@yahoo.com

**Background:** Mouse mammary tumor virus (MMTV) is thought to have a role in human breast cancer (BC) pathogenesis. BRCA1 and 2 genes mutations are well-established risk factors for BC. The purpose of this study was to evaluate the presence of MMTV in familial and non-familial Egyptian breast cancer patients. We also aimed to establish a correlation between BRCA genes mutations and MMTV infection in those patients.

**Patients and Methods:** The study was included 80 BC patients and 10 healthy women were included as a control group. We used PCR to amplify a 250-bp MMTV-like env sequence. We also used PCR followed by direct sequencing to identify the genetic variation of exons 2, 13, 19 of BRCA1 gene and exon 9 and region f of exon 11 of BRCA2 gene. High resolution melting (HRM) analysis was used to screen the selected exons of BRCA1/2 genes in order to detect different variants.

**Results:** MMTV DNA-like env sequences were detected in 70%, 76% of familial and non-familial BC patients, respectively, and it was not detected in any of the control subjects. The presence of viral sequences was associated with larger tumor size in the sporadic patients. Seventy BC patients showed variations in BRCA1/2 genes according to HRM analysis and sequencing analysis showed two different sequences of polymorphism among 22 familial and non-familial BC patients.

**Conclusion:** MMTV DNA was present among BC patients and it was associated with increased tumor growth. This indicates a potential role for MMTV in BC patients with and without deleterious mutation in BRCA1/2 genes.

**Keywords:** breast cancer, MMTV, BRCA1/2, HRM, Egypt

## Introduction

Breast cancer (BC) is the deadliest cancer among women in several countries representing a quarter of cancer cases diagnosed in women.<sup>1,2</sup> In Egypt, BC represents 32% of women's cancers.<sup>3</sup>

Studying of BC has improved its detection and treatments leading to a significant decrease in the BC-associated death.<sup>4</sup> However, little is known about the main causes, etiology, or carcinogenesis of the BC due to its great complexity. BC is highly heterogeneous comprising several types and subtypes with distinct genetic background, pathogenesis, response to treatment, and prognosis indicating the presence of other causative agents that may be implicated in BC carcinogenesis. Genetic mutations play a role in the multi-step process of BC malignancy.<sup>5-7</sup> In addition, somatic mutations are also involved in the familial BC

cases.<sup>6</sup> BC cases are mostly sporadic, also are associated with a low frequency of familial predisposition. Therefore, relying on identifying genetic causes will only explain a limited number of BC cases.<sup>8</sup> In 2017, it was reported that 154,794 women living with metastatic breast cancer (MBA) in the United States indicating the increase of MBA cases in USA.<sup>9</sup> Besides, environmental factors such as alcohol consumption, smoking, lifestyle, obesity, and body mass index affect the BC incidence and prognosis.<sup>10–13</sup> Moreover, infection is another risk factor for BC development. Infection plays a role in 16% of human cancers.<sup>14</sup> These cancers are caused by viral oncogenic genes such as human papilloma virus (HPV) which cause cervical cancer, or by viruses that diminish the host immune response such as human immunodeficiency virus (HIV), or by viruses that cause chronic inflammation and damage such as HCV and HBV. Interestingly, mouse mammary tumors were caused by Mouse Mammary Tumor Virus (MMTV) which is transmitted in breast milk.<sup>15,16</sup> Viral infections play a role in the etiology and pathogenesis of BC such as HPV, polyomavirus, and MMTV.<sup>17,18</sup> The MMTV-like *env* gene sequences of the MMTV-like virus, previously known as mouse milk factor, were identified at a significantly high level in breast cancers compared to the non-cancer tissues.<sup>19–21</sup> MMTV-like virus was identified in 30–74% of BC tissues in several countries.<sup>16,22–24</sup> MMTV initiates BC via the insertional mutagenesis process which causes uncontrolled cellular proliferation leading to cancer initiation and progression.<sup>16</sup>

Genetic mutations increase the BC susceptibility among certain families, especially the tumor suppressor genes such as breast cancer anti-estrogen resistance-1 and 2 (BRCA1 and BRCA2), which are found to be frequently mutated in hereditary cancers.<sup>5–7</sup> BRCA1 and BRCA2 mutations account for 30% of breast and ovarian cancers.<sup>25</sup> The estimated cumulative breast cancer risk at age 70 years was 0.46 in BRCA1 women and 0.43 in BRCA2 women.<sup>26</sup> However, the role of MMTV in BC initiation and progression in patients with BRCA mutations is not completely understood.

Up to our knowledge, there is no report linking the role of MMTV and BRCA1 and 2 mutations in the BC development among Egyptian women. In this study, we tested the presence of MMTV-like gene among Egyptian BC patients and correlated between MMTV-like gene, BRCA1 and two genetic mutations, and clinicopathological parameters of BC.

## Patients and Methods

### Patients

The study is done on BC patients (n=80) admitted to surgery, and pathology departments, National Cancer Institute, Cairo University, Egypt between March 2011 to July 2013, it is a retrospective analysis. BC patients, enrolled in this study, were divided into two groups; group 1: BC patients with family history (n=30) and group 2: sporadic BC patients (n=50). The inclusion criteria were women suffering from BC and did not receive any chemotherapy treatment before surgery. BC women received chemotherapy prior to surgery were not included in this study. Ten (n=10) healthy women served as controls. The control subjects were age-matched with the BC groups. The sample size was selected based on Al Dossary et al,<sup>20</sup> MMTV *env* proviral sequences were detected in 5.9% (6/101) of breast cancer tissues in a group of Saudi women. Based on these findings, a sample size of 80 patients with breast cancer is required for estimating the expected proportion with 5% absolute precision and 95% confidence. The study was approved by the Institution Review Board (IRB) of the National Cancer Institute, Cairo University (IRB No. 200801002.2). A written informed consent was obtained from each participant. All of the experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

### Methods

#### Sample Collection and Storage

Breast tumor tissues and blood were collected from each BC patient and healthy controls and were stored at  $-80^{\circ}\text{C}$  until use. Total genomic DNA was extracted from blood samples using QIAamp DNA Mini kit (Qiagen, Hilden, and Germany), according to the manufacturer's instructions. Tumor tissues were processed as described previously.<sup>27</sup> The concentration and quality of the extracted DNA were determined by Nanodrop ND2000 spectrophotometer (Nanodrop Technologies, USA).

### Molecular Detection

#### Nucleic Acid Extraction

DNA was extracted from breast cancer tissues using the phenol-chloroform method as previously described.<sup>27</sup> MTV DNA extraction from MCF-7 cell line was done using QIAamp DNA extraction kit (Qiagen, Valencia, USA) according

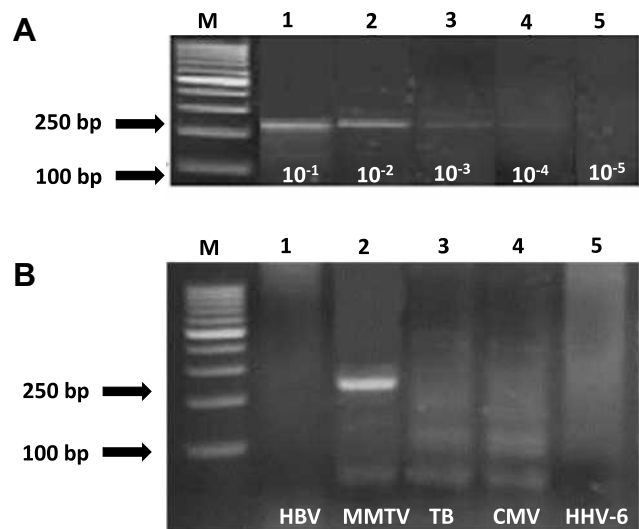
to the manufacturer's instructions. MCF-7 was obtained from VACSERA (Egypt). The concentration and quality of MMTV DNA were assessed by spectrophotometry using a Nano-Drop 2000 spectrophotometer. About 100 ng of DNA template was used as a positive control in each PCR run.

## Detection of MMTV-DNA

Detection of MMTV-DNA was done using primers targeting *env* genomic region targeting the nucleotide position from 1386 to 1640 bp of MMTV genome (GenBank ID AF346816). The PCR reaction was done as described previously<sup>28</sup> at the following conditions: 94°C for 5 minutes, followed by 35 cycles of: 95°C for 1–5 min, 55°C for 1 min, and 72°C for 5 min, with a final step at 72°C for 10 minutes. The PCR product was run on a 2.5% agarose gel. The expected PCR product size appeared at 250 bp.

## Sensitivity and Specificity of MMTV PCR Assay

The sensitivity of MMTV PCR assay was evaluated by making serial dilutions of positive control (MMTV extracted from MCF-7 cell line) from  $10^{-1}$  up to  $10^{-5}$  (Figure 1). The specificity of the PCR assay was assessed by testing the PCR reaction against other pathogens such as HBV, TB, CMV, and HHV-6 (Figure 1).



**Figure 1** Sensitivity and specificity of PCR assay for detection of MMTV DNA-like *env* sequences extracted from MCF-7 cell line. **(A)** Ethidium bromide-stained gel electrophoresis shows sensitivity assay of primers used to amplify MMTV DNA-like *env* sequences. Positive signals are 250 bp. Lane M = 100-bp ladder; lanes 1–5 = positive lanes of serial dilutions from  $10^{-1}$ – $10^{-5}$ . **(B)** Ethidium bromide-stained gel electrophoresis of specificity assay of primers used to amplify MMTV DNA-like *env* sequences. Positive signal at 250 bp. For MMTV DNA product (lane 2), lanes 1, 3, 4, 5 are negatives, Lane M = 100bp ladder.

## HRM Assay of BRCA1/2 Variations and Sequencing for the Variants

PCR reactions were done to amplify the exons (2, 13, and 19) of BRCA1 and exons (9 and 11f) of BRCA2 using sets of primers previously described<sup>29</sup> and mentioned in Table 1, using Applied Biosystems 7500 Fast Real-Time PCR system, USA (SN.275014218, Singapore). The PCR and HRM were performed in a single run on a 7500 Fast Real-Time PCR System in a reaction mix containing MeltDoctor HRM Master Mix (ThermoFisher, applied biosystem, Vilnius, Lithuania), 200 nM of each PCR primers and 20 ng of genomic DNA. The PCR reaction was run as the followings: an activation step at 95°C for 10 minutes followed by 55 cycles of 95°C for 10 seconds, a touch down of 65°C to 55°C for 10 seconds (1°C/cycle) and 72°C for 30 seconds. The products were heated to 95°C for 1 minute prior to the high-resolution melting step and the HRM was carried out over the range from 72°C to 95°C rising at 1°C per second with 30 acquisitions per degree. The melting curves obtained from control samples (healthy women, n=10) were used for normalization and comparison with BC samples. Analysis of the obtained curves was performed using Applied Biosystems HRM v2.0.2 software. The HRM software plots fluorescence signal over temperature. BRCA1 and BRCA2 PCR products which show variations by HRM analysis were sequenced (Macrogen, Korea). The PCR products were sequenced using the same primers used for amplification reactions.

## Statistical Analysis

Data were analyzed using SPSS advanced statistics version 20 (SPSS Inc., IBM, Chicago, IL) and GraphPad Prism 6 (La Jolla, California). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For paired qualitative data, comparison was done using McNemar test. For quantitative data, comparison between two groups was done using either Student's *t*-test or Mann–Whitney test. *P*-value < 0.05 was considered significant.

## Results

The mean age of the familial and non-familial BC groups was  $47.0 \pm 9.4$  and  $50.9 \pm 11.4$ , respectively ( $p=0.113$ ). The

**Table 1** List of Primers Used to Amplify the Selected Exons from BRCA1 and 2 Genes

Gene Accession No.	Primers	Nucleotide Sequence	Nucleotide Positions	Amplicon Size
<b>BRCA1</b> MIM# 113705	Exon 2	F: 5'- CTT TTA AAA AGA TAT ATA TAT ATG TTT TTC TAA TGT GT-3' R: 5'- TCC CAA ATT AAT ACA CTC TTG TGC TGA-3'	93 826–93 863 93 999–93 981	173bp
	Exon 13	F: 5'- GAT GTC TAC AAT TTC ACC TTT CT-3' R: 5'- TTG CCA AAA TGA CGA ACA CA-3'	138 557–138 579 138 690–138 669	133bp
	Exon 19	F: 5'- CTT TCT CTT ATC CTG ATG GGT TGT G-3' R: 5'- GAG TGG TGG GGT GAG ATT TTT GTC-3'	160 808–160 833 160 978–160 955	170bp
<b>BRCA2</b> MIM# 600185	Exon 9	F: 5'- ATA AGG GGG GAC TAC TAC TAT ATG TG-3' R: 5'- CAA AAA AAC CTG TAG TTC AAC TAA ACA GAG-3'	20 368–20 393 20 588–20 558	220bp
	Exon 11f	F: 5'- GCA GGA TTT TAA TTC AAA CCA TAA TTT AAC AC3' R: 5'- CCC TAA ACC CCA CTT CAT TTT CAT C3'	27 158–27 189 27 528–27 504	371bp

mean tumor size was significantly larger in patients with non-familial BC than in familial BC patients (5.7±3.9 cm vs 4.3±2.6 cm, respectively,  $p=0.031$ ). Clinicopathological characteristics including age, estrogen receptor status (ER), progesterone receptor status (PR), lymph node involvement, safety margin among familial and non-familial BC are presented in Table 2. Positive ER was significantly higher among non-familial BC compared to familial BC (84% vs 63.3%, respectively,  $p=0.036$ ). Most of the familial BC patients were premenopausal, while the majority of non-familial BC patients were postmenopausal ( $p=0.033$ ).

### Presence of MMTV DNA in All Studied Groups

Sensitivity and specificity of qualitative PCR assay for detection of MMTV extracted from MCF-7:

The sensitivity level of the assay was found to be approximately 100 copies/ $\mu$ L (Figure 1A). The PCR was found to be highly specific for MMTV DNA as none of the other tested viruses were amplified (HBV, CMV, TB, and HHV6) (Figure 1B). MMTV-DNA-like *env* sequences were detected in 38 of 50 (76%) of non-familial BC tissues and in 21 of 30 (70%) of familial BC tissues ( $p=0.555$ ) (Table 3). It was not detected in the 10 healthy control women.

### MMTV DNA-Like *env* Sequences and Clinicopathological Factors

The presence of MMTV-DNA like *env* sequences was not associated with different clinicopathological factors in

both BC groups except larger tumor size in the non-familial group ( $p=0.012$ ) (Table 3).

### Detection of BRCA1 and 2 Gene Variations Using HRM

The aligned melt curve graph and difference plots for the selected exons of BRCA1/2 genes are shown in (Figure 2). Variations in BRCA1/2 genes in the selected exons are summarized in (Table 4). Patients with familial BC showed more frequent variations (2–4) in exon 13 of BRCA1 gene compared to non-familial BC (50% vs 26%,  $p=0.029$ ). In contrast, significantly more frequent variations (2–4) (same as the previous comment) in exon 9 of BRCA2 gene were noticed in the non-familial BC compared to familial BC (62% vs 33%,  $p=0.013$ ) Table 4. Forty-two samples that showed HRM variations were subjected to sequencing analysis to identify the type of mutations.

### Mutational Analysis of BRCA Genes Using Sequencing Assay

Only 22/42 (52%) familial and non-familial BC patients had changes in exons 13, 19 of BRCA1 gene and exon 11f of BRCA2, whereas exon 2 of BRCA1 and exon 9 of BRCA2 did not demonstrate any changes in their sequences. Sequencing analysis of exon 11f of BRCA2 gene showed nine different sequences, submitted to gene bank for annotation under the following accession numbers and entry ID: LC331907, LC331908, LC331909, LC331910, LC331911, LC331912, LC331708, LC331709, LC331710. Regarding, LC331909 and LC331911, SNP rs1801206 was detected at



**Table 2** Clinicopathological Features in Breast Cancer Patients

Parameters	Non-Familial Breast Cancer N= 50 (%)	Familial Breast Cancer N=30 (%)	p-value
<b>Age (years)</b>			0.16
<45	16 (32.0)	14 (46.7)	
≥45	34 (68.0)	16 (53.3)	
<b>Tumor Size (Mean±SD)</b>	5.7±3.9	4.3±2.6	0.031
<b>ER-status</b>			0.036
Positive	42 (84)	19 (63.3)	
Negative	8 (16)	11 (36.7)	
<b>PR-Status</b>			0.48
Positive	37 (74)	20 (66.7)	
Negative	13 (26)	10 (33.3)	
<b>Lymph Node Involvement</b>			0.67
Positive	38 (76)	24 (80)	
Negative	12 (24)	6 (20)	
<b>Safety Margin</b>			0.75
Positive	4 (8)	3 (10)	
Negative	46 (92)	27 (90)	
<b>Tumor Grade</b>			1.00
G I, II	45 (90)	27 (90)	
G III	5 (10)	3 (10)	
<b>Pathological Type</b>			0.44
Invasive duct carcinoma	46 (92)	26 (86.7)	
Invasive lobular carcinoma	4 (8)	4 (13.3)	
<b>Menopausal Status</b>			0.033
Premenopausal	21 (42)	20 (66.7)	
Postmenopausal	29 (58)	10 (33.3)	
<b>Laterality</b>			0.25
Right	31 (63.3)	15 (50)	
Left	18 (36.7)	15 (50)	
<b>Stage</b>			0.54
II	25 (50)	19 (63.3)	
III	21 (42)	10 (33.3)	
IV	4 (8)	1 (3.3)	
<b>Hormonal Therapy</b>			0.119
Yes	41 (82)	20 (66.7)	
No	9 (18)	10 (33.3)	

anchored position 27,272 of NG\_012772.3 on chromosome 13 (A/C/G) (Table 5, Figure 3A). LC331912 demonstrated SNP rs1799952 at anchored position 27,392 of NG\_012772.3 on chromosome 13 (A/G/T).

As for sequence analysis of exon 13, 11 variations were detected in the same sequences, but we could not process them for annotation due to low quality of data; further studies will be performed on this exon. Our results showed one sequence in exon 19, has annotated with accession number and entry ID of LC368290. This sequence demonstrated prominent SNP rs571834423, detected at anchored position 43,057,061 of NC\_000017.11 on chromosome 17 (Table 5, Figure 3B).

Only one sporadic BC patient showed mutation in exon 19 of BRCA1 and was associated with negative ER.

## Relation Between MMTV-Like env Sequence, BRCA1/2 Genes Polymorphism and Clinicopathological Factors

MMTV DNA was present in all BC patients who demonstrated polymorphism in exons 19 of BRCA1 and exon 11f of BRCA2 genes. Most BC patients were >45 years and most of them were positive ER (>75%). Distribution of data is presented in Table 6.

## Discussion

BC is classified into several types and subtypes which differ significantly in its pathogenesis, response to treatment, and prognosis. Genetic mutations, especially in BRCA1 and 2 genes significantly increase the risk of BC development. Viral infections play a role in BC pathogenesis in several populations. However, there have not been any studies that linked the viral infection and genetic mutations in the development of BC in Egyptian patients. So, we thought to be the first study to investigate this critical matter.

The origin of MMTV has not been well identified but it is strongly thought to be transmitted from mice to humans.<sup>30</sup> The incidence of MMTV among BC patients is variable.<sup>30–33</sup> The incidence of BC is higher in areas where certain types of mice with rich MMTV in its genome, *Mus domesticus*, are common.<sup>22,30</sup> MMTV is capable of infecting human cells, suggesting MMTV transmission to humans.<sup>34</sup>

**Table 3** MMTV-Like *env* Sequence and Its Association with Clinicopathological Features in Breast Cancer Patients

	Non-Familial		Familial	
	N= 50 (%)		N=30 (%)	
	MMTV		MMTV	
	Positive N=38(76)	Negative N=12(24)	Positive N=21(70)	Negative N=9(30)
<b>Age (years)</b>				
<45	12 (75)	4(25)	11(78.6)	3(21.4)
≥45	26(76.5)	8(23.5)	10(62.5)	6(37.5)
<b>p-value</b>	0.91		0.44	
<b>ER Status</b>				
Positive	31 (73.8)	11(26.2)	14(73.7)	5(26.3)
Negative	7 (87.5)	1 (12.5)	7(63.6)	4(36.4)
<b>p-value</b>	0.406		0.563	
<b>PR Status</b>				
Positive	29 (78.4)	8(21.6)	13(65)	7(35)
Negative	9 (69.2)	4 (30.8)	8(80)	2(20)
<b>p-value</b>	0.506		0.398	
<b>Tumor Size</b>				
<5cm	14 (60.9)	9 (39.1)	12 (66.7)	6 (33.3)
≥5cm	24 (88.9)	3 (11.1)	9(75)	3 (25)
<b>p-value</b>	<b>0.012</b>		0.626	
<b>Lymph Node</b>				
Positive	30 (78.9)	8(21.1)	17 (70.8)	7(29.2)
Negative	8 (66.7)	4(33.3)	4(66.7)	2(33.3)
<b>p-value</b>	0.385		1.000	
<b>Tumor Grade</b>				
G I, II	33(73.3)	12(26.7)	19(70.4)	8(29.6)
G III	5(100)	0(0)	2(66.7)	1(33.3)
<b>p-value</b>	0.319		1.000	
<b>Safety Margin</b>				
Positive	4(100)	0 (0.0)	2(66.7)	1(33.3)
Negative	34(73.9)	21(26.1)	19(70.4)	8(29.6)
<b>p-value</b>	0.56		1.000	
<b>Pathological Type</b>				
Invasive duct carcinoma	35 (76.1)	11(23.9)	17(65.4)	9(34.6)
Invasive lobular carcinoma	3(75)	1 (25)	4 (100)	0(0.0)
<b>p-value</b>	1.000		0.287	
<b>Menopausal Status</b>				
Premenopausal	15(71.4)	6(28.6)	14(70)	6(30)
Postmenopausal	23(79.3)	6(20.7)	7(70)	3(30)
<b>p-value</b>	0.52		1.000	

(Continued)

Table 3 (Continued).

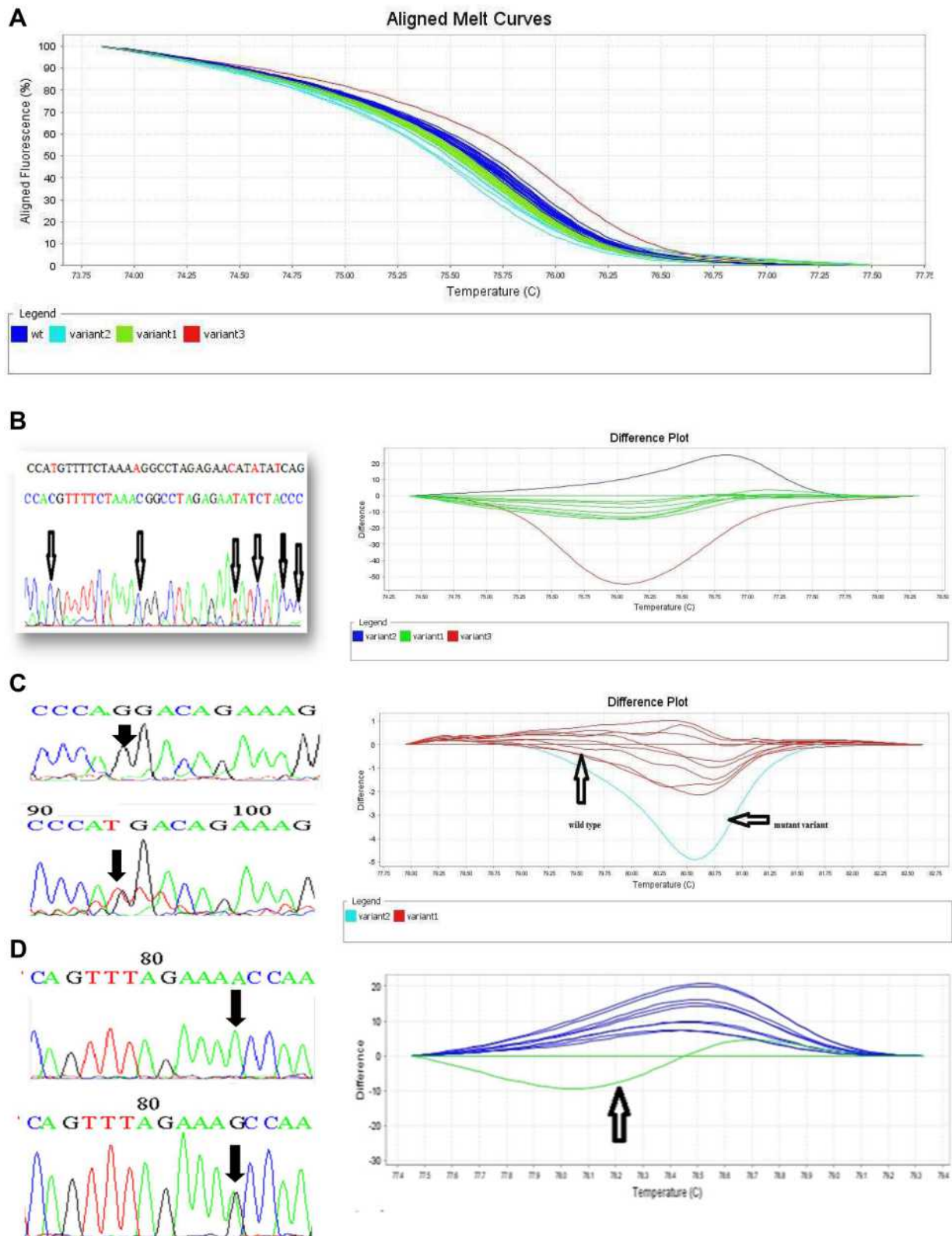
	Non-Familial		Familial	
	N= 50 (%)		N=30 (%)	
	MMTV		MMTV	
	Positive N=38(76)	Negative N=12(24)	Positive N=21(70)	Negative N=9(30)
<b>Laterality</b>				
Right	24(77.4)	7(22.8)	12(80)	3(20)
Left	13(72.2)	5(27.8)	9(60)	6(40)
<b>p-value</b>	0.683		0.427	
<b>Stage</b>				
II	18(72)	7(28)	13(68.4)	6(31.6)
III+IV	20(80)	5(20)	8(72.7)	3(27.3)
<b>p-value</b>	0.508		0.804	
<b>Hormonal Therapy</b>				
Yes	8(88.9)	1(11.1)	5(50)	5(50)
No	30 (73.2)	11(26.8)	16(80)	4(20)
<b>p-value</b>	0.317		<b>0.091</b>	

Our results show that MMTV DNA-like *env* sequences were detected in 70% and 76% in familial and non-familial BC tissues, respectively. This prevalence is considerably higher than the prevalence rates reported elsewhere.<sup>22,28,33,35,36</sup> MMTV-like sequences have been found in human breast cancer tissues in wide geographical variations (0.8–74%).<sup>24,35</sup> Several studies have demonstrated the presence of MMTV-like *env* sequences in 30–40% of BC cases in several Western countries including the United States, Italy, Mexico, Brazil, Argentina, and Australia.<sup>22</sup> In Southeast Asia, where BC incidences are known to be of low rate, the presence of MMTV-like *env* sequences in BC was 12% in Japan and 10–17% in China.<sup>37</sup> However, in Vietnamese BC has been reported to be only 0.8% MMTV-like positive.<sup>24,33</sup> In Tunisia, BC was reported to have a high proportion of MMTV-like *env* sequences, almost 74%,<sup>24</sup> but later MMTV-like *env* sequences were reported at a lower rate.<sup>23</sup>

PEV positive tumors were reported to be more aggressive compared to PEV negative tumors and most tumors containing MMTV-like sequences are considered aggressive tumors, which propose a link between MMTV-like and inflammatory BC.<sup>24</sup> Therefore, the difference in MMTV prevalence between our study and that reported elsewhere could be explained by a higher frequency of inflammatory BC in our patients. Especially that a higher

rate of inflammatory BC was reported in Egypt compared to other countries.<sup>38</sup> The MMTV-like prevalence reported here concurs with other studies performed in countries with a high incidence of BC such as the United States which reports MMTV-like sequences in 30–40% of BC patients.<sup>39–41</sup> Other studies from Argentina, Italy, and Australia report MMTV-like prevalence in 31.7%, 37.7%, and 42.2% of BC tumors, respectively.<sup>28,33,36</sup> However, studies from other countries could not identify MMTV-like sequences in any cases.<sup>42–44</sup> This discrepancy could be largely attributed to variation in detection procedures or tissue heterogeneity.<sup>22</sup> When comparing PEV-positive tumors with PEV-negative tumors in the latter series, a higher proportion of aggressive tumors contained the MMTV-like sequences, suggesting a correlation between MMTV-like and inflammatory BC. Importantly, MMTV-like *env* sequences could not be identified in any of the tissues obtained from apparently healthy women included in this study.

We could not establish a correlation between MMTV DNA-like *env* sequences and any clinicopathological parameters of BC such as age, hormonal receptors, histologic subtype, or tumor grade, similar findings were reported elsewhere.<sup>45</sup> However, our results show a significant association between the presence of MMTV DNA-like *env* sequences and larger tumor size (>5cm) in



**Figure 2** HRM results and its sequencing analysis. **(A)** Aligned melt curves of BRCA1 gene exon 2 by HRM. 2a. Dark blue: wt (wild type), light blue: variant 2, green: variant1, and red: variant 3 **(B)** HRM results and its sequencing analysis amplifying 133bps of exon 13, BRCA1 gene. Dark blue: variant 2, green: variant 1, red: variant 3 **(C)** HRM results and its sequencing analysis amplifying 170bps of exon 19, BRCA1 gene. Red: wild type and light blue: mutant variant **(D)** HRM results and its Sequencing analysis amplifying 371bps of BRCA2 gene exon 11 of BRCA2 gene. Dark blue: wild type and green: mutant variant.



**Table 4** Frequency of Variations in the Selected Exons of BRCA1/2 Genes in Breast Cancer Patients as Detected by HRM Assay

	Non-Familial		Familial		p-value
	N=50 (%)		N=30 (%)		
	Variant	Variants	Variant	Variants	
	(1)	(2-4)	(1)	(2-4)	
<b>BRCA1</b>					
Exon 2	31(62)	19(38)	23(76.7)	7(23.3)	0.175
Exon 19	30(60)	20(40)	18(60)	12(40)	1.000
Exon 13	37(74)	13 (26)	15(50)	15(50)	0.029
<b>BRCA2</b>					
Exon 9	19(38)	31(62)	20(66.7)	10(33.3)	0.013
Exon 11 F	39(78)	11(22)	25(83.3)	5(16.7)	0.564

non-familial BC patients. This indicates the detection of MMTV DNA as a risk factor for BC alongside age, menopausal status, hormonal receptor.<sup>46</sup> Nonetheless, it cannot be considered as major risk factors that significantly affect BC pathogenesis, since we could not find any significant difference in presence of MMTV DNA-like *env* sequences between familial and non-familial BC. It has been reported before that MMTV is correlated with tumor grade in BC, where MMTV-like *env* sequences were found in 26.3% of ductal carcinoma *in situ* and 53.8% of infiltrating ductal carcinoma.<sup>33</sup> In addition, MMTV identified in BC tumors was associated with inflammatory tumors compared to tumors without MMTV-like *env* sequences.<sup>23</sup> To this end, large-scale studies are required to rule out whether MMTV is truly correlated with specific clinicopathological parameters that complicate BC carcinogenesis.

Genetic factors are risk factors for BC susceptibility, specifically BRCA1 and BRCA2 gene mutations. Identification of founder and recurrent mutations in those

genes could be a major step towards the improvement of genetic counseling. In BC, such improvements largely rely on identifying relevant mutations present in certain ethnic groups, this will eventually lead to developing rapid and less expensive tests for the determined human population.<sup>47</sup> In this regard, high resolution melting (HRM) assay has proven to be an attractive choice of mutation screening; it is rapid, sensitive, and relatively inexpensive.<sup>48,49</sup> The current study investigated the sequence variation of exons 2,13 and 19 of BRCA1 and exons 9 and certain regions of exon 11 (11f) of the BRCA2 gene in Egyptian familial and non-familial BC patients.

Sequencing analysis was performed on a total of 70 familial and non-familial BC patients who showed sequence variations as detected by HRM assay. HRM results showed a frequency of variants in exon 13 of the BRCA1 gene to be more prominent among familial patients, whereas the frequency of variants in exon 9 of the BRCA2 gene was more evident among the sporadic cases. This establishes HRM as

**Table 5** Polymorphism in BRCA1/2 Genes

Program Database	Accession Number	Refseq Genome	Anchored Position	BRCA/ Exon	Allele	Variant	No. of Observation
BLASTN Human G+T	LC331909 LC331911	NG_012772.3	27,272	BRCA2/11	A/C/G	rs1801406	2
BLASTN Human G+T	LC331912	NG_012772.3	27,392	BRCA2/11	A/G/T	rs1799952	2
BLASTN Human G+T	LC368290	NC_000017.11	43,057,061	BRCA1/19	C/T/A	rs571834423	1



Figure 3 Polymorphism of BRCA1 and BRCA2. (A) Blast result of LC331909 (exon 11 of BRCA2) using NG\_012772.3 demonstrated SNP. (B) Blast result of LC368290 (exon 13 of BRCA1) using NC\_000017 demonstrated SNP.

**Table 6** Presence of MMTV, BRCA Variations by HRM and Some Clinical Parameters Among 80 Women with Breast Cancer Disease. (A) In Familial Breast Cancer. (B) In Non-Familial Breast Cancer

<b>(A)</b>										
<b>Parameters</b>	<b>Familial Breast Cancer Patients N=30</b>									
	<b>HRM Variant (1)</b>					<b>HRM Variants (2–4)</b>				
	<b>BRCA1 Exons</b>			<b>BRCA2 Exons</b>		<b>BRCA1 Exons</b>			<b>BRCA2 Exons</b>	
	<b>2 N=23</b>	<b>13 N=15</b>	<b>19 N=18</b>	<b>9 N=20</b>	<b>11f N=25</b>	<b>2 N=7</b>	<b>13 N=15</b>	<b>19 N=12</b>	<b>9 N=10</b>	<b>11f N=5</b>
<b>Age (years)</b>										
<45 (N=14)	12	6	10	9	13	2	8	4	5	1
≥45 (N=16)	11	9	8	11	12	5	7	8	5	4
<b>ER-status</b>										
Pos. (N=19)	14	9	12	12	16	5	10	7	7	3
Neg. (N=11)	9	6	6	8	9	2	5	5	3	2
<b>MMTV</b>										
Pos. (N=21)	15	10	11	13	16	6	11	10	8	5
Neg. (N=9)	8	5	7	7	9	1	4	2	2	0
<b>(B)</b>										
<b>Parameters</b>	<b>Non-Familial Breast Cancer Patients N=50</b>									
	<b>HRM Variant (1)</b>					<b>HRM Variants (2–4)</b>				
	<b>BRCA1 Exons</b>			<b>BRCA2 Exons</b>		<b>BRCA1 Exons</b>			<b>BRCA2 Exons</b>	
	<b>2 N=31</b>	<b>13 N=37</b>	<b>19 N=30</b>	<b>9 N=19</b>	<b>11f N=39</b>	<b>2 N=19</b>	<b>13 N=13</b>	<b>19 N=20</b>	<b>9 N=31</b>	<b>11f N=11</b>
<b>Age</b>										
<45 (N=14)	20	25	21	12	27	13	9	12	21	7
≥45 (N=16)	11	12	9	7	12	6	4	8	10	4
<b>ER-status</b>										
Pos. (N=19)	26	31	24	14	33	16	8	14	28	9
Neg. (N=11)	5	6	6	5	6	3	5	6	3	2
<b>MMTV</b>										
Pos. (N=21)	22	31	23	14	33	16	8	15	24	4
Neg. (N=9)	9	6	7	5	6	3	5	5	7	7

a sensitive, cost-effective and rapid tool for the detection of genetic mutations in BC patients. Results showed that 42 of 70 (60%) BC patients showed different types of polymorphisms. The BRCA1 gene did not show the classical mutation 185delAG instead it showed a variation at rs571834423 at the position of 43,057,061 of NC\_000017.11. Similarly, exon 9 of the BRCA2 gene did not show the previously reported mutation (3492InsT).<sup>50</sup> Instead, polymorphism in BRCA2

exon 11 (rs1801406 and rs1799952) was observed in 12% of BC patients. Eleven of 42 (26%) BC patients showed the same changes in exon 13 but could not be analyzed.

Our results showed that MMTV-like *env* sequences are present in BC, independent of BRCA1 and 2 variations; however, the fact that it is present in patients with polymorphisms highlights it as a potential risk factor, but this observation remains to be further verified. In light of our findings, the

sequences of BRCA genes have not been completely studied in Egyptian patients and further analysis of these genes is urgently required as they harbor different genetic backgrounds than those reported in other populations. Genetic variation in BRCA genes could be playing a role in determining response to treatment and BC prognosis.

Until now, the biological implication of MMTV-like env sequences in BC remains largely unknown, and whether these sequences are of no importance to BC etiology and maybe just an epiphenomenon needs to be further verified. On the other hand, the fact that MMTV-like env sequences are only detected in cancerous and not normal breast tissues suggests MMTV-like as a risk factor for BC. In addition, results from this study show a link between MMTV-like sequences and larger tumor size which supports a role for MMTV-like in BC. In this regard, viral infections, along with hormones and some genetic variations, were suggested as contributing factors in a multifactorial model for BC; our findings support this model.<sup>51</sup>

## Conclusion

In conclusion, our findings show a high prevalence of MMTV-like env sequences among Egyptian BC patients and not in healthy women. In addition, MMTV-like was correlated with increased tumor size, which suggests MMTV as a risk factor for BC. Also, our results confirm the importance of reducing viral sequences from Egyptian BC patients compared to other populations. This increase in MMTV-like env sequences suggests higher exposure to the virus. Finally, the genetic sequence of BRCA genes in our patients showed a different genetic variation than that reported in other BC patients, which suggest further analysis of whole functional sequences of these genes. Finally, we report the presence of MMTV-like env sequences in BC patients with genetic polymorphisms which indicate a role of these sequences in BC carcinogenesis.

## Ethical Approval

The research has been approved by National Cancer Institute, Cairo University, IRB No. 200801002.2.

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## Disclosure

The authors report no conflicts of interest in this work.

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