

Intervirolology

Intervirolology , DOI: 10.1159/000525277

Received: November 25, 2021

Accepted: April 15, 2022

Published online: May 31, 2022

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ISSN: 0300-5526 (Print), eISSN: 1423-0100 (Online)

<https://www.karger.com/INT>

Intervirolology

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Research Article

Incidence of HPVs, EBV and MMTV-like Virus in Breast Cancer in Qatar

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Short Title: HPV, EBV and MMTV-like virus in Breast Cancer in Qatar

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Number of Tables: 3

Number of Figures: 1

Word count: 1,930

Keywords: HPV; EBV; MMTV-like virus; breast cancer; Qatari population

Abstract

Introduction: Human papillomaviruses (HPVs), Epstein-Barr virus (EBV), and mouse mammary tumor virus-like virus (MMTV-like virus) can be present and contribute to breast cancer development and progression. However, the role of these oncoviruses and their crosstalk in breast cancer is still unclear.

Methods: We explored the co-presence of high-risk HPVs, EBV, and MMTV-like virus in 74 breast cancer samples from Qatar using PCR.

Results: We found the presence of HPV and EBV in 65% and 49% of our cancer samples cohort; 47% of the samples are positive for both oncoviruses. The MMTV-like virus alone was detected in 15% of the samples with no significant association with clinicopathological features. The three oncoviruses were co-present in 14% of the cases; No significant association was noted between the co-presence of these viruses and the clinicopathological features.

Conclusion: Despite the presence of the oncoviruses, additional studies are necessary to understand their interactions in human breast carcinogenesis.

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Introduction

Breast cancer is the second most common cancer worldwide, including the Middle East (ME) region and Qatar [1]. In comparison to ME countries, Qatar has the highest incidence of breast cancer, accounting for approximately 38% of all new cancer cases [2, 3]. Intriguingly, in comparison with their counterparts in the West, women in the ME are affected with breast cancer at a relatively younger age (<50 years) [3]. Moreover, women with breast cancer in the ME region usually display advanced-stage disease and a more aggressive phenotype [4]. In addition to genetic and environmental factors, oncogenic viruses, mainly high-risk human papillomaviruses (HPVs), Epstein-Barr virus (EBV), and mouse mammary tumor virus-like virus (MMTV-like virus) are found in various types of human cancers, including breast [5-9].

High-risk HPVs are double-stranded DNA viruses that can immortalize human mammary epithelial cells. More specifically, E5 and E6/E7 oncoproteins of HPV cooperate in human cancer pathogenesis [10, 11]; in this context, it has been demonstrated that E6/E7 oncoproteins can convert non-invasive and non-metastatic breast cancer cells into invasive and metastatic phenotypes via their cooperation with other oncogenes [12-16]. Several studies reported that HPVs could be co-present with other strains of human oncoviruses (EBV and MMTV-like virus) and cooperate with these oncoviruses to initiate and/or enhance carcinogenesis in various types of human cancers, including breast [17-24]. EBV, a double-stranded DNA gamma-herpes virus, creates a persistent infection in memory B-lymphocytes with latent EBV infection (EBV nuclear antigen 1 (EBNA1), and/or latent membrane protein 2: LMP2 [25]; latent EBV may reactivate and stimulate viral genes (*EBNAs*, *LMP1*, and *LMP2*) inducing epithelial cell growth, proliferation, and angiogenesis while inhibiting apoptosis [26-28]. Earlier studies reported that EBV is present in several human cancers, including breast [29]. The mouse mammary tumor virus-like virus (MMTV-like virus), a part of the Betaretroviridae family, is a non-acutely transforming retrovirus [30]. MMTV-like virus was previously found in several human cancers, including breast [8, 9]; however, the role of MMTV-like virus in the onset of cancer remains unclear.

While several studies have reported the presence/co-presence of high-risk HPVs, EBV, and MMTV-like virus in human breast cancer [21, 22, 24], other investigations could not confirm this [31-33]. Thus, we herein explored the presence/co-presence of these oncoviruses in breast cancer samples from Qatari women.

Materials and Methods

Breast cancer samples

This investigation used formalin-fixed, paraffin-embedded (FFPE) breast cancer samples obtained from patients who received surgical treatment at Hamad General Hospital in Qatar, as described previously [22]. A cohort of 74 FFPE blocks (punch samples of 2 mm thickness) from pathologically confirmed invasive breast cancer patients was used for DNA extraction [22]. None of the patients received neoadjuvant treatment modalities (chemotherapy, radiotherapy, endocrine therapy, immunotherapy, targeted therapy). In addition, we included 14 control samples from another cohort (normal/benign breast tissues) [34]. The study was approved by the Ethical Committees of Qatar University and Hamad Medical Corporation (QU-IBC-2018/22; HMC:24-2-2019, Doha, Qatar).

Oncogenic Viral detection by PCR

The detection and genotyping of high-risk HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, and 58) and EBV was performed as previously described [22]. In addition, PCR was performed for MMTV-like virus (Forward primer: 5'-GATGGTATGAAGCAGGATGG-3' and Reverse primer: 5'-CCTCTTTTCTATATCTATTAGCTGAGGTAATC-3'). For all reactions, GAPDH was used as an internal control. Analyses were completed as illustrated earlier [22]. The Invitrogen Platinum II Hot-Start Green PCR Master Mix (2X) (ThermoFisher Scientific, Waltham, MA, USA) was used to perform PCR. Briefly, the MMTV-like virus gene was amplified for an initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s. The samples were finally incubated for 10 min at 72 °C for a final extension. HPV and EBV genes were amplified as described previously [22]. The PCR product was resolved using 1.5% agarose gel electrophoresis. For each experiment performed, respective positive and negative controls were used; positive control for HPV included

normal oral epithelial (NOE) cell line expressing E6/E7, for EBV diluted DNA plasmids for EBNA1 and LMP1, while for MMTV-like virus, we used MDA-MB-231 as the positive control [35]. Nuclease-free water instead of DNA was used for the negative control [15].

Statistical analysis

Statistical analysis and plotting of graphs were done using GraphPad Prism software (version 8.4.3). To determine a significant association between the presence/co-presence of HPVs, EBV and MMTV-like virus with clinicopathological data (Nottingham histological grade, tumor stage, and lymph node involvement), we performed Chi-square (χ^2) test with Yates' correction and Fisher's exact test in addition to Wilcoxon test. Data were calculated as non-parametric files, and statistically significant results were achieved if p-values were ≤ 0.05 .

Results

In this study, we investigated the presence of MMTV-like virus in addition to its co-presence with high-risk HPVs and EBV in a cohort of 74 breast cancer samples from the Qatari population and 14 normal/benign breast tissues. We recently revealed that 65% of the samples were positive for high-risk HPVs, while 36/74 (49%) of the samples were positive for EBV [22]; in addition, we found that high-risk HPVs and EBV are co-present in (47%) of breast cancer cases [22].

We found that 11/74 (15%) of breast cancer cases are positive for MMTV-like virus (Fig. 1). In addition, 11/74 (15%) and 10/74 (14%) showed co-presence of HPV and MMTV-like virus as well as EBV and MMTV-like virus, respectively (Fig. 1). More significantly, we report that 10/74 (14%) of the breast cancer samples co-expressed all three oncoviruses (Fig. 1). On the other hand, normal/benign breast samples lacked the co-presence of HPV and MMTV-like virus ($p = 0.2$), EBV, MMTV and HPV, and EBV and MMTV-like virus in normal breast tissues ($p = 0.3$, each, respectively).

Concerning the clinicopathological characteristics, in our previous study, we found that the co-presence of high-risk HPVs is associated with tumor grade ($p = .04$) and stage ($p = .04$) in comparison with HPV positive alone and/or EBV positive alone in addition to HPV/EBV negative [22]. In the current study, we did not find any significant associations between the presence of MMTV-like virus alone with patients' age ($p = .74$), tumor grade ($p = .25$), tumor stage ($p = .12$), hormonal receptors (ER and PR) status ($p = .68$ and $p = .56$, respectively), and HER-2 status ($p = .06$) (Table 1). In addition, there was no significant association of the co-presence of high-risk HPVs, EBV and MMTV-like virus with clinicopathological parameters (patients' age, tumor grade, tumor stage, ER status, PR status and HER-2 status) ($p = .93$, $p = .25$, $p = .12$, $p = .79$, $p = .7$ and $p = .21$, respectively) (Table 2).

Discussion

This is the first study on the presence/co-presence of high-risk HPVs, EBV and MMTV-like virus in human breast cancer samples in the Gulf region.

Our data revealed HPV positivity in 65% and 36% of our breast cancer and normal/benign tissue samples, with no significant difference in the HPV positivity between breast cancer and the controls. On the other hand, we found EBV positivity in 49% of breast cancer cases in Qatari women, while normal/benign breast tissue samples lacked EBV presence. In addition, we did not note any significant association of either HPV or EBV positivity with the clinicopathological outcomes in breast cancer cases.

Previous studies reported the presence of the *MMTV* gene in human breast cancer patients with varying prevalence ranging from 0 to 74% [33, 36-42] (Table 3). Our study revealed that MMTV-like virus is present in 15% of breast cancer samples in the Qatari cohort, with no presence in normal/benign breast samples (0%). Similar findings were reported in other Middle East countries [36-42] (Table 3). Variations in prevalence detection may be attributed to technical and methodological variations among studies, and retroviral etiology needs to be explored further [33]. The presence of MMTV-like virus supports our findings suggesting a plausible role in breast carcinogenesis. Katz et al. [43] demonstrated that proteins expressed by the MMTV-like virus envelope gene could transform normal breast epithelial cells. Moreover, insertion of the MMTV proviral DNA in the vicinity of proto-oncogenes (Wnt-1 and Fgf) enhanced cell growth [44] and the development of mammary tumors [45].

Although the role of viral co-infection in breast cancer is still controversial, it is postulated that MMTV infection can trigger the expression of latent DNA viruses, including HPV or EBV, to initiate the onset and development of cancer. We previously reported that the co-presence of high-risk HPVs and EBV (47% of the breast cancer samples) is associated with advanced tumor stage and grade ($p = .04$) in breast cancer [20]. In the present study, we report the co-infection of HPV and MMTV-like virus in 15% of our cohort, while EBV and MMTV-like virus co-incidence was found in 14% of Qatari breast cancer patients. Moreover, 14% of the samples were positive for all three viruses. Even though the prevalence of co-infection of HPV, EBV and MMTV-like virus is infrequent, we postulate that a synergistic carcinogenic effect of HPVs, EBV and MMTV-like virus exists and can play a role in the onset and progression of several human cancers, including breast, *via* the initiation and/or enhancement of the epithelial-mesenchymal transition event as previously reported by several studies [11, 20, 29, 46, 47]. On the other hand, Rickinson [48] demonstrated that co-infection with these viruses might trigger chronic inflammation due to the release of inflammatory cytokines, which provides a favorable microenvironment for viral infection.

Concordant to other findings, in this study, we did not find any association between the presence of MMTV-like virus and its co-presence with HPV or EBV and clinicopathological characteristics [37, 49-51]. However, other studies reported a significant association between MMTV-like virus and clinicopathological features [37, 52]. Nevertheless, we believe that further studies are needed to evaluate the role of MMTV-like virus and its crosstalk with other oncoviruses in a larger breast cancer cohort.

The present study has several limitations. Firstly, we had a small sample size; therefore, a larger cohort from Qatar and other ME countries is needed to confirm our findings. Secondly, an appropriate control group of normal/benign samples from the same patients/population was lacking. Finally, the reported data relied on a single methodology (PCR assay for all three viruses).

Nevertheless, our previous studies exploring the presence of HPV and EBV in different types of cancers using PCR and IHC generated comparable results [19, 34, 53-55].

Conclusion

We herein report for the first time the presence/co-presence of high-risk HPVs, EBV, and MMTV-like virus in breast cancer in the Qatari population without significant correlation with clinicopathological parameters. Finally, the data from the current study indicates that breast cancer incidence and progression could be reduced using available vaccines against oncoviruses and their associated diseases.

Statements

Acknowledgment

We would like to thank Mrs. A. Kassab for her critical reading of the manuscript.

Statement of Ethics

“The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. Patient consent was waived as this study used existing FFPE specimens. The study was approved by the Ethical Committees of Qatar University and Hamad Medical Corporation (QU-IBC-2018/22; HMC:24-2-2019, Doha, Qatar).”

Conflict of Interest Statement

“The authors have no conflicts of interest to declare.”

Funding Sources

The current study was supported by the grants from Qatar University: QUCP-CMED-2021-1 and QUCP-CMED-22/23-529

Author Contributions

“**Conceptualization**, Halema F Al-Farsi, Hamda Al-Thawadi and Ala-Eddin Al Moustafa; **Methodology**, Ishita Gupta; **Formal analysis**, Ishita Gupta and Semir Vranic; **Resources**, Reem Al-Sarraf, Hanan Fargahly, Ali A. Sultan, Halema F Al-Farsi and Ala-Eddin Al Moustafa; **Data curation**, Reem Al-Sarraf, Hanan Fargahly, Semir Vranic and Ishita Gupta; **Writing—original draft preparation**, Ishita Gupta; **Writing—review and editing**, Semir Vranic, Ali A. Sultan, Halema F Al-Farsi, Hamda Al-Thawadi and Ala-Eddin Al Moustafa; **Supervision**, Semir Vranic, Halema F Al-Farsi, Hamda Al-Thawadi and Ala-Eddin Al Moustafa; **Funding acquisition**, Semir Vranic, Halema F Al-Farsi, Hamda Al-Thawadi and Ala-Eddin Al Moustafa.

All authors have read and agreed to the published version of the manuscript.”

Data Availability Statement

All data generated or analyzed during this study are included in this article (and/or) its supplementary material files. Further inquiries can be directed to the corresponding author.

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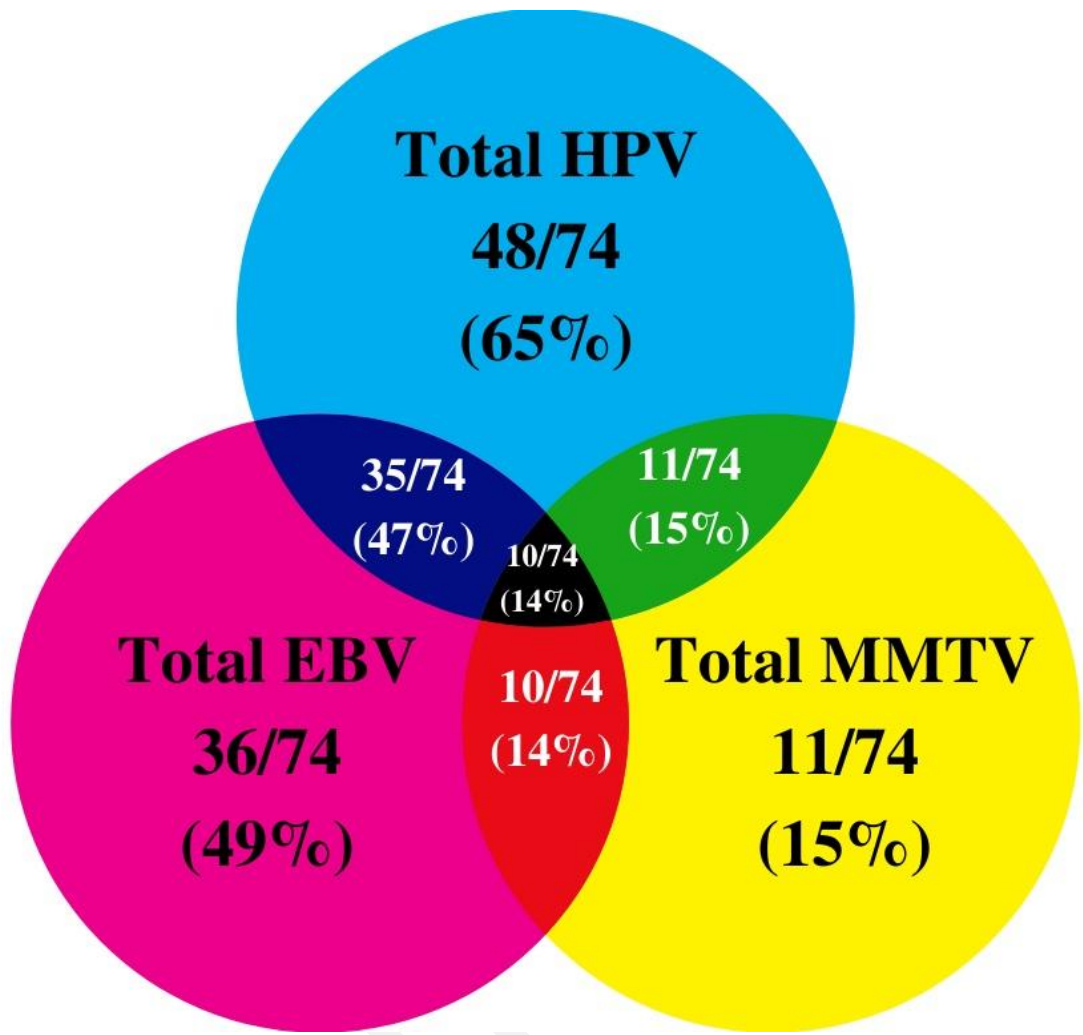
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Figure Legends

Fig. 1. Venn diagram depicting single and multiple infections with HPV, EBV and/or MMTV-like virus in Qatari breast cancer samples (n=74).

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Tables

Table 1. Correlation between clinicopathological characteristics and MMTV-like virus positivity

Age			
Age (years)	MMTV-like virus Positive (%)	MMTV-like virus Negative (%)	<i>p</i> -value
≤50	3 (4)	22 (30)	0.74
>50	8 (11)	41 (55)	
Total	11 (15)	63 (85)	
Nottingham Histological Grade			
Grade			<i>p</i> -value
I	4 (5.5)	10 (14)	0.25
II	4 (5.5)	36 (50)	
III	3 (4)	15 (21)	
Total	11 (15)	61 (85)	
Tumor Stage			
Stage			
Early Stage (I-II)	6 (13)	33 (70)	0.57
Advanced Stage (III-IV)	0 (0)	8 (17)	
Total	6 (13)	41 (87)	
Estrogen Receptor (ER) Status			
ER+	9 (12)	50 (70)	0.68
ER-	2 (3)	11 (15)	
Total	11 (15)	61 (85)	
Progesterone Receptor (PR) Status			
PR+	7 (10)	47 (65)	0.57
PR-	4 (5)	14 (20)	
Total	11 (15)	61 (85)	
HER-2 Status			
HER-2+	5 (7)	11 (15)	0.06
HER-2-	6 (8)	50 (70)	
Total	11 (15)	61 (85)	

Comparison was made between presence/absence of HPV (HPV+/HPV-) and clinicopathological characteristics.

*indicates significant *p*-values ($p < 0.05$).

Tables

Table 2. The correlation between clinicopathological characteristics and HPV/EBV/MMTV-like virus status (the HPV/EBV/MMTV-like virus positive group was compared with other subgroups of breast cancer with various HPV/EBV/MMTV-like virus status)

Age			
Age (years)	HPV+/EBV+/MMTV-like virus+ (%)	HPV±/EBV±/MMTV-like virus± (%)	<i>p-value</i>
≤50	3 (4)	22 (30)	0.93
>50	7 (9)	42 (57)	
Total	10 (13)	64 (87)	
Nottingham Histological Grade			
Grade			<i>p-value</i>
I	4 (5.5)	10 (14)	0.25
II	4 (5.5)	36 (50)	
III	2 (3)	16 (22)	
Total	10 (14)	62 (86)	
Tumor Stage			
Stage			
Early Stage (I-II)	6 (13)	33 (70)	0.12
Advanced Stage (III-IV)	0 (0)	8 (17)	
Total	6 (13)	41 (87)	
Estrogen Receptor (ER) Status			
ER+	8 (11)	51 (71)	0.79
ER-	2 (3)	11 (15)	
Total	10 (14)	62 (86)	
Progesterone Receptor (PR) Status			
PR+	7 (10)	47 (65)	0.7
PR-	3 (4)	15 (21)	
Total	10 (14)	62 (86)	
HER-2 Status			
HER-2+	4 (6)	12 (17)	0.21
HER-2-	6 (8)	50 (69)	
Total	10 (14)	62 (86)	

HPV+/EBV+/MMTV-like virus+ denotes a co-presence of HPV, EBV and MMTV-like virus. HPV±/EBV±/MMTV-like virus± indicates a combination of HPV+/EBV-/MMTV-like virus- (HPV presence/EBV absence/MMTV-like virus absence), HPV-/EBV+/MMTV-like virus- (HPV absence/EBV presence/MMTV-like virus absence), HPV-/EBV-/MMTV-like virus+ (HPV absence /EBV absence/MMTV-like virus presence), and HPV-/EBV-/MMTV- (lack of all the three HPV, EBV and MMTV-like virus).

*indicates significant *p*-values (<0.05).

Tables

Table 3. Prevalence of MMTV-like virus in breast cancer in Middle-East populations

Country	Year	MMTV-like virus (%)
Egypt	2021	Sporadic breast cancer tissues (38/50, 76%) Familial breast cancer tissues (21/30, 70%)
Saudi Arabia	2018	Breast cancer tissues (6/101, 6%) Non-cancerous adjacent tissues (9/93, 10%) Control breast tissue obtained from individuals without cancer (0/51, 0%)
Iran	2017	Breast carcinomas (19/59, 32%) Non-malignant breast tissue samples (3/59, 5%)
Morocco	2014	24/42 (57%)
Tunisia	2008	17/122 (14%)
Egypt	2005	3/23 (13%)
Tunisia	2004	28/38 (74%)