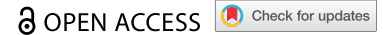


RESEARCH PAPER



Presence of high-risk HPVs, EBV, and MMTV in human triple-negative breast cancer

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ABSTRACT

Breast cancer, the most frequent disease amongst women worldwide, accounts for the highest cancer-related mortality rate. Triple-negative breast cancer (TNBC) subtype encompasses ~15% of all breast cancers and lack estrogen, progesterone, and HER2 receptors. Although risk factors for breast cancer are well-known, factors underpinning breast cancer onset and progression remain unknown. Recent studies suggest the plausible role of oncoviruses including human papillomaviruses (HPVs), Epstein-Barr virus (EBV), and mouse mammary tumor virus (MMTV) in breast cancer pathogenesis. However, the role of these oncoviruses in TNBC is still unclear. In the current study, we explored the status of high-risk HPVs, EBV, and MMTV in a well-defined TNBC cohort from Croatia in comparison to 16 normal/non TNBC samples (controls) using polymerase chain reaction assay. We found high-risk HPVs and EBV present in 37/70 (53%) and 25/70 (36%) of the cases, respectively. The most common HPV types are 52, 45, 31, 58 and 68. We found 16% of the samples positive for co-presence of high-risk HPVs and EBV. Moreover, our data revealed that 5/70 (7%) samples are positive for MMTV. In addition, only 2/70 (3%) samples had co-presence of HPVs, EBV, and MMTV without any significant association with the clinicopathological variables. While, 6/16 (37.5%) controls were positive for HPV ($p = .4$), EBV was absent in all controls (0/16, 0%) ($p = .01$). In addition, we did not find the co-presence of the oncoviruses in the controls ($p > .05$). Nevertheless, further investigations are essential to understand the underlying mechanisms of multiple-oncogenic viruses' interaction in breast carcinogenesis, especially TNBC.

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Introduction





Breast cancer is the second most common cancer worldwide, and it affects more than two million women accounting for around half a million deaths annually.¹ Breast cancer is classified into four molecular subtypes (Luminal A, Luminal B, HER2 + and triple-negative breast cancer (TNBC));^{2,3} of which the TNBC subtype accounts for around 12–17% of all breast cancer cases.⁴ TNBC is one of the most aggressive subtypes^{4,5} and lacks hormonal expression of estrogen and progesterone (ER and PR) as well as HER-2/neu receptor.⁶ In addition, TNBC frequently affects younger patients and is characterized by high tumor grade, advanced stage, and higher Ki-67 proliferative index.^{7,8}


Along with environmental and genetic factors, it is estimated that around 20% of human cancers are associated with infectious agents, including oncoviruses, especially high-risk human papillomaviruses (HPVs), Epstein-Barr virus (EBV), mouse mammary tumor virus (MMTV) and human mammary tumor virus (HMTV) which can be plausibly involved in the onset and progression of different types of human carcinomas.^{9–13}

HPV, a small epitheliotropic, non-enveloped, double-stranded DNA virus, infects the epithelial cells (epidermal or mucosal) and can prompt neoplastic transformation (both

benign and malignant) of infected cells.^{14,15} The HPV genome consists of eight open reading frames (ORFs) and is divided into three functional elements (the early (E), and late (L) regions, and a long control region (LCR)).¹⁶ While the L region encodes the structural proteins (L1-L2) and is involved in virus assembly,¹⁶ the high-risk HPV early proteins (E5, E6, and E7) act as oncogenes and modulate molecular mechanisms of infected cells, including the deregulation of epithelial-mesenchymal transition (EMT) biomarkers.^{17,18} Indeed, various studies have reported the etiological role of HPVs in head and neck,¹⁹ cervical,²⁰ bladder,²¹ and breast²² carcinomas.

Likewise, EBV, a double-stranded DNA gamma-herpes virus, infects 90% of the adult population.^{23,24} The EBV genome spans over 184-kbp and encodes over 85 genes including viral oncogenes such as six EBV-encoded nuclear antigens (EBNA1, -2, -3A, -3B, -3 C and -LP) and latent membrane proteins (LMP1, -2A, and -2B) in addition to numerous non-coding RNAs (EBERs and miRNAs).²⁴ EBV infection is involved in the onset of various epithelial carcinomas, comprising nasopharyngeal (NPC), breast, cervical, and gastric

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cancer.²⁵ EBNA1 and LMP1 are considered oncogenic proteins of EBV that can induce cellular proliferation and motility, inhibit apoptosis and, enhance cell invasion and angiogenesis.^{26,27}

On the other hand, the mouse mammary tumor virus (MMTV) belongs to the Betaretroviridae family²⁸ and is presently considered a frequent etiologic causal factor for T-cell lymphomas and breast cancer in mice.^{29,30} Earlier studies identified homologous antigens and sequences to MMTV, known as MMTV-like sequences in human biological samples, including breast milk,³¹ saliva,³² and peripheral blood lymphoid cells.³³ Similar to the role of MMTV in the onset of tumors (lymphomas, renal adenocarcinoma, salivary gland, and mammary tumors) in mice,³⁴ MMTV-like virus was detected in biliary cirrhosis as well as in various human cancers, including lymphoma, endometrial, liver, ovary, prostate, skin, lung and breast.^{12,13} However, the role of MMTV in these cancers is not clearly defined yet.

Earlier investigations have indicated the presence/co-presence of high-risk HPVs, EBV, and MMTV-like virus in human breast cancer worldwide.^{35–38} However, a few studies failed to detect the presence of these oncoviruses in human breast cancer as well as normal mammary tissues.^{39–41} Based on previous reports depicting the role of HPVs, EBV, and MMTV-like virus in the initiation of various cancers, comprising breast,^{19,30,36–38,42–45} we explored the presence/co-presence of high-risk HPVs, EBV, and MMTV-like virus in TNBC samples in Croatian women.

Materials and methods

Sample collection and DNA extraction

The study included 72 formalin-fixed paraffin-embedded (FFPE) tissue samples initially diagnosed as TNBC at the Ljudevit Jurak Clinical Department of Pathology and Cytology, Sestre milosrdnice University Hospital Center, Zagreb, Croatia.

All cases were negative for estrogen receptor (ER; clone EP1, Dako), progesterone receptor (PR; clone 636, Dako), and HER-2/neu receptor (Hercep Test, monoclonal Rabbit, anti-Human; HER2 protein, Dako). ER, PR and HER-2/neu were scored using the ASCO/CAP guidelines.^{46,47} The tumors were graded following the Nottingham Histologic Score system.⁴⁸

In addition, fourteen normal/benign breast tissues from another breast cohort were used for comparison.⁴⁹ For the study, all cases were de-identified and patients' information anonymized. A local ethical committee (institutional review board) of the Sestre Milosrdnice University Hospital Center approved the study (number of approval: EP-13659/21-11).

Before molecular assays, all cases were re-reviewed by a board-certified pathologist (S.V.) to confirm the diagnosis and select appropriate FFPEs for PCR assays.

DNA was extracted from FFPE tissue samples using the Thermo Scientific GeneJET FFPE DNA Purification Kit according to the manufacturer's instructions (ThermoFisher Scientific, USA) and as previously described.³⁶ Briefly, FFPE sections underwent enzymatic digestion using 200 µl of digestion buffer followed by lysing and release of genomic DNA

using Proteinase K solution (20 µl); the DNA was de-crosslinked by heat incubation (90°C) for 40 min. The supernatant containing DNA was obtained from the solution and mixed with 200 µl binding buffer followed by the addition of ethanol (96%); the lysate was loaded onto the purification column, then, adsorbed DNA was washed (wash Buffers 1 and 2) to eliminate contaminants and finally eluted with elution buffer (60 µl).

Polymerase chain reaction analysis

Purified genomic DNA from each sample was analyzed for HPVs, EBV, and MMTV-like virus by polymerase chain reaction (PCR) using specific primers for high-risk HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68 as well as EBNA1, EBNA2 (Forward primer: 5'-AGGGATGCCTGGACACAAGA-3' and Reverse primer: 5'-TTGTGACAGAGGTGACAAAA-3') and LMP1 of EBV as previously described.^{50,51} In addition, PCR was performed for MMTV (Forward primer: 5'-GATGGTATGAAGCAGGATGG-3' and Reverse primer: 5'-CCTCTTTTCTCTATATCTATTAGCTGAGGTAATC-3'). GAPDH was used as an internal control. All the primers and analyses were performed as previously described.⁴⁹ In each experiment, negative control (instead of DNA, nuclease-free water) and positive control (HPV: HeLa cell line for L1 region⁵² and normal oral epithelial (NOE) cell line expressing E6/E7 of HPV type 16 for E6/E7 region;⁵³ EBV: Diluted DNA plasmids for EBNA1, EBNA2, and LMP1 of EBV; MMTV-like virus: MDA-MB-231⁵⁴) were used.

PCR was performed using the Invitrogen Platinum II Hot-Start Green PCR Master Mix (2X) (ThermoFisher Scientific, Waltham, MA, USA). Briefly, HPV and EBV genes were amplified for an initial denaturation at 94°C for 2 min followed by 40 cycles of 94°C for 30 s, annealing at temperatures ranging from 50°C to 62°C for 30 s depending on each primer's melting temperature as previously described,^{50,51} and 72°C for 30 s. In parallel, EBNA2 and MMTV-like virus genes were 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. The PCR product from each exon was resolved using 1.5% agarose gel electrophoresis.

The samples expressing all three oncoproteins, EBNA1, EBNA2, and LMP1, were taken into consideration to determine EBV positivity in our cohort.

Statistical analysis

GraphPad Prism software (version 8.4.3) was used to carry out all the statistical and plotting of graphs. To assess the significance of HPVs, EBV, and MMTV-like virus associations with clinicopathological data (Nottingham histological grade, tumor stage, and lymph node involvement) in correlation with the presence/co-presence of these oncoviruses, we utilized Chi-square (χ^2) test with Yates' correction and Fisher's exact test. The results were considered statistically significant if p -values were ≤ 0.05 in two-tailed tests.

Results

Clinicopathological characteristics of the cohort

The clinicopathological characteristics of the TNBC cohort are summarized in Table 1. The mean age of all patients is 62.4 years (standard deviation (SD), ± 14.7 years).

The status of high-risk HPVs, EBV, and MMTV and their association with clinicopathological characteristics

After closer examination of the 72 samples used for PCR assessment of high-risk HPVs, EBV, and MMTV-like viruses (Figure 1), we found that two samples were not TNBC but

Table 1. Clinicopathological characteristics of patients with triple-negative breast cancer (n = 70).

Characteristic	Categories	Number (%)
Age	≤ 50	18 (25%)
	> 50	53 (74%)
	Unknown	1 (1%)
Nottingham Histological Grade	I	0 (0%)
	II	11 (16%)
	III	59 (84%)
Tumor (pT) Stage	pT1	22 (31%)
	pT2	34 (49%)
	pT3	4 (6%)
	pT4	9 (13%)
	Unknown	1 (1%)
Lymph Node (pN) status	Positive	21 (30%)
	Negative	49 (70%)

malignant phyllodes tumor and fibrocystic disease of the breast. These samples along with 14 normal/benign breast tissue samples were compared with the TNBC samples.

Thirty-seven of the remaining 70 TNBC samples in our cohort are positive for high-risk HPVs (52.8%) (Figure 2 and Table 2); the most commonly present high-risk HPVs are HPV52 (18%) followed by HPV45 (11%), HPV31, and HPV58 (4%, each) and HPV68 (1%) (Figure 3). Notably, HPV types 16, 18, 33, 39, 51, 56, 59, and 66 were not detected in our cohort (Figure 3). Furthermore, our data revealed that while only 27/70 (39%) of the cases are positive for only one HPV subtype, 10/70 (14%) cases are positive for two HPV subtypes.

On the other hand, our data revealed that 25/70 of the samples are positive for EBV (36%) (Figure 2 and Table 2); of these 25 cases, EBNA1, EBNA2, and LMP1 of EBV are individually present in 55/70 (78%), 31/70 (44%) and 0/70 (0%) of the TNBC cases, respectively. Additionally, normal/non TNBC samples exhibited positivity for high-risk HPVs (6/16, 37.5%), the most frequent HPV subtypes present in normal/non TNBC samples include HPV52 (6/16, 37.5%) and HPV58 (1/16, 6.3%) (one case co-expressed HPV52 and HPV58); no significant difference was observed in HPV-positivity between normal/non-TNBC and TNBC samples ($p = .4$). In addition, PCR analysis showed that EBV (EBNA1, EBNA2 and LMP1) was absent in all normal/non TNBC samples (0/16, 0%). However, it is important to highlight a significant difference in EBV-positivity

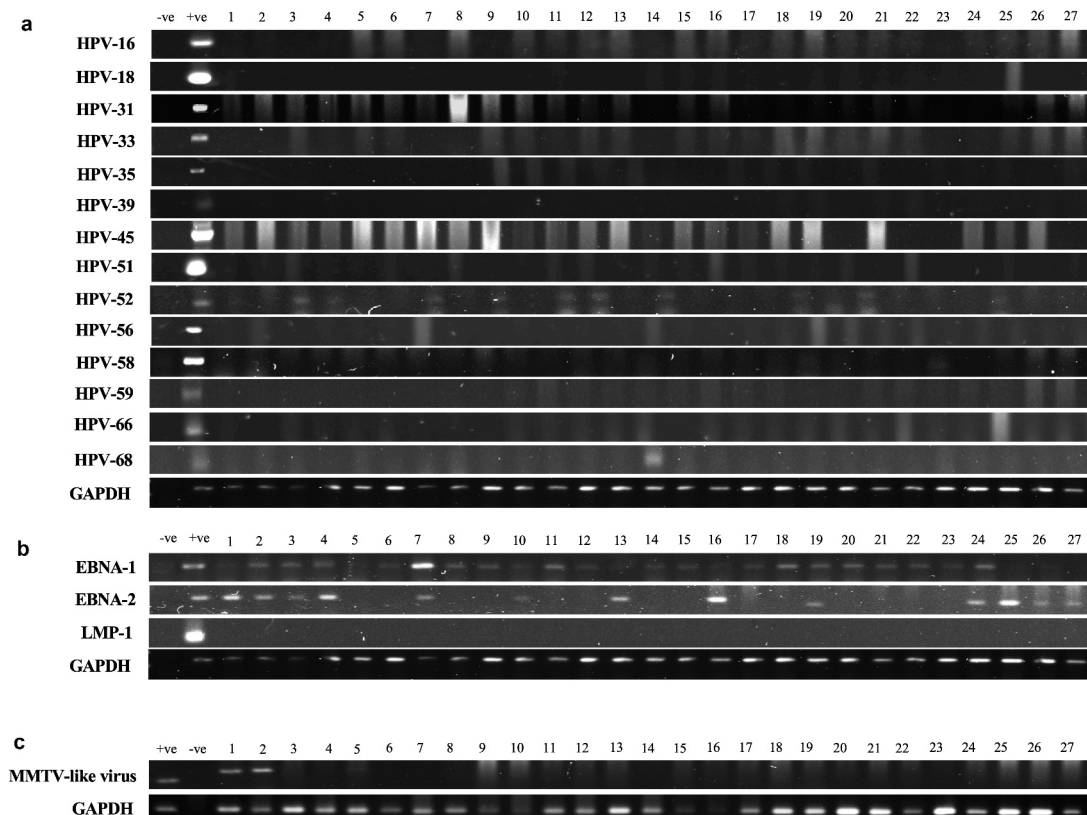


Figure 1. (a–c). Representative PCR reactions for (a) high-risk HPV-subtypes, (b) EBV (EBNA1, EBNA2 and LMP1) and (c) MMTV-like virus in 27 different samples of TNBC. GAPDH was used as an internal control. GAPDH was run common for both HPV and EBV. Respective positive (+ve) and negative (-ve) controls were used as described in the materials and methods section; L- GeneRuler 100 bp DNA Ladder (ThermoFisher Scientific, USA).

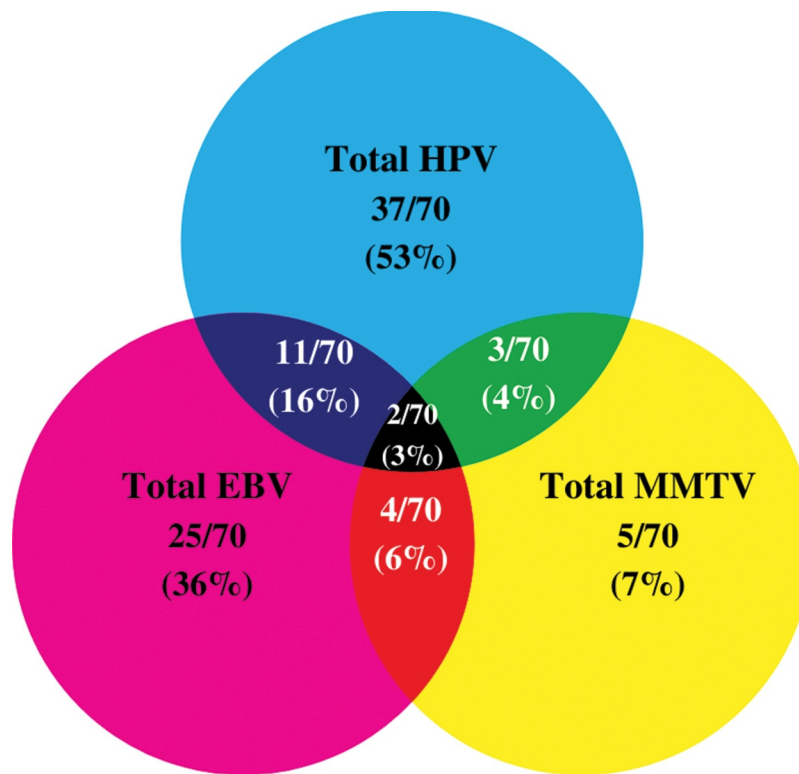


Figure 2. Venn Diagram depicting single and multiple infections with HPV, EBV, and/or MMTV-like virus in Croatian triple-negative breast cancer (TNBC) samples ($n = 70$).

Table 2. Presence of single infection with HPV, EBV, or MMTV-like virus in 70 samples of TNBC.

Single infection		
HPV+	EBV+	MMTV-like virus+
53%	36%	7%

between normal/non-TNBC and TNBC samples ($p = .01$). More significantly, our data revealed the co-presence of high-risk HPVs and EBV in 16% (11/70) of TNBC cases (Figure 2); however, we did not find any significant correlation between EBV and various HPV types. Moreover, co-presence of high-risk HPVs and EBV was not detected in normal/non-TNBC samples (0/16, 0%); there was no significant difference between normal/non-TNBC and TNBC samples ($p = .2$).

In parallel, we found 5/70 (7%) of the TNBC cases to be positive for MMTV-like virus (Figure 2 and Table 2) with no presence in normal/non-TNBC samples (0/16, 0%) ($p = .6$). In addition, 3/70 (4%) and 4/70 (6%) showed co-presence of HPV and MMTV-like virus as well as EBV and MMTV-like virus, respectively. Only two TNBC cases (3%) co-expressed all three oncoviruses (Figure 2). On the other hand, while, we did not find the co-presence of HPV and MMTV-like virus (0/16, 0%), EBV and MMTV-like virus (0/16, 0%) and HPV, EBV and MMTV-like virus (0/16, 0%) in normal/non TNBC samples, there was no significance between normal/non-TNBC and TNBC samples ($p = .9$, each, respectively).

However, we did not find significant associations between the clinicopathological parameters (tumor grade, tumor stage, and lymph node involvement) and the presence/co-presence of high-risk HPVs, EBV, and MMTV-like virus.

Discussion

To the best of our knowledge, this is the first study on the presence/co-presence of high-risk HPVs, EBV, and MMTV-like virus in TNBC from Croatia. Our study revealed that 53% of the samples are positive for these oncoviruses. Numerous studies have reported the presence of high-risk HPVs in human breast cancer patients, including TNBC worldwide, with prevalence ranging from 4% to 86% (Table 3a).^{22,55–59}

Our data are similar to studies performed in a large cohort (>1000 samples) of cervical cancer patients from Croatia; the studies have reported HPV prevalence ranging from 43% to 64%.^{60–62} Moreover, a study by Sabo et al. demonstrated that 31.5% of the samples had only a single HPV infection while the prevalence of multiple HPV infections was 10%;⁶⁰ this is in line with our data that revealed 38% of the cases were positive for only one HPV subtype and 16% of the cases positive for two HPV subtypes. Similar to our data, HPV prevalence in breast cancer in Spanish women was 52%,⁶³ while in Italian women, the prevalence was 25%.⁶⁴ On the other hand, in the UK, HPV prevalence of 42% was reported in breast cancer;⁶⁵ in Germany, HPV prevalence in breast cancer was 86%.⁶⁶ On the other hand, in the Italian TNBC cases, HPVs were detected in 15% of the samples,⁶⁷ while only

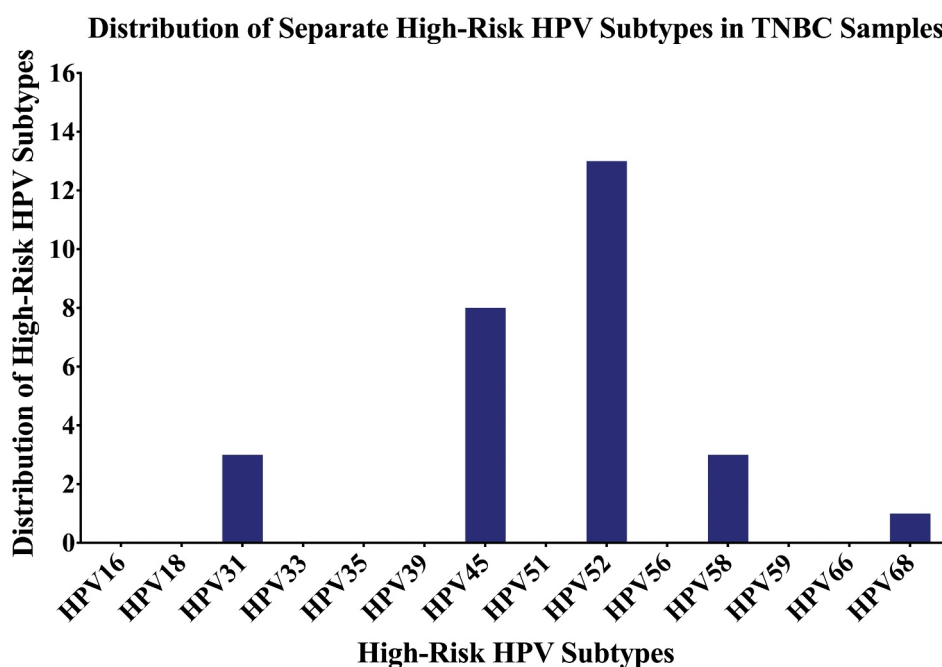


Figure 3. Distribution of each high-risk HPV subtype in Croatian triple-negative breast cancer (TNBC) samples. PCR analysis included 70 TNBC samples revealing that the most frequent HPV subtypes are HPV52, HPV45, HPV31, HPV58, and HPV68.

Table 3. (a) Prevalence of HPV in TNBC patients in different populations. (b) Prevalence of EBV in TNBC patients in different populations.

Country	Year	HPV (%)	Reference
(a)			
Qatar	2020	3/10 (30%)	36
Morocco	2018	4/9 (44%)	70
Spain	2017	11/24 (9%)	63
India	2017	37/67 (55%)	69
Australia	2015	1/2 (50%)	68
Spain	2015	0/16 (0%)	120
Venezuela	2015	2/2 (100%)	59
Algeria	2014	5/25 (20%)	95
Italy	2014	6/40 (15%)	67
(b)			
Qatar	2020	4/10 (40%)	36
France	2015	8/38 (21%)	94
Algeria	2014	6/25 (24%)	95

9% of the TNBC cases in Spain were positive for HPVs.⁶³ This discrepancy can be attributed to the smaller cohort in the Italian and Spanish studies. Concordant with our data, studies from Australia, India, and Morocco reported HPVs prevalence of 50%,⁶⁸ 55%,⁶⁹ and 44%⁷⁰ in TNBC samples, respectively. Nevertheless, our recent study in Qatar found HPV positivity in 30% of TNBC cases.³⁶

Indeed, one of the key findings in our study is the predominance of HPV genotypes 52 and 45 followed by 31, 58, and 68 in TNBC samples from Croatian women. It is important to emphasize that HPV types 16 and 18 are the most frequently expressed genotypes in cancers worldwide,^{57,58,71,72} in Croatia as well, the most prevalent subtype in cervical cancer was HPV16 (44%), followed by HPV18 (39%), HPV51 (18%), HPV31 (11%), HPV33 (8%), and HPV45 (3%).⁷³ Another study in cervical cancer by Sabol et al., found HPV16 as the most prevalent type (61%), followed by HPV18 (10%), HPV31 (4%), HPV33 (4%),

HPV35 (2%), HPV45 (6%), HPV52 (3%) and HPV58 (2%).⁶⁰ However, in our study, we did not detect HPV types 16 and 18 in our cohort from Croatia. A study in China also failed to detect HPV16 and 18 in breast cancer samples.⁷⁴ However, in breast cancer samples from Australia, HPV18 was detected as the most prevalent subtype in breast cancer specimens.⁶⁸ This variation in HPV prevalence and genotype distribution can be attributed to geographical location, sample size, type of cancer, and methodological differences.^{58,75} Interestingly, HPV52 phylogenetically resembles HPV31 (*HPV16* species)⁷⁶ and is the most common HPV-type detected in our samples. Likewise, HPV58 is phylogenetically close to HPV33 (*HPV16* species)⁷⁶ and was the third most common subtype detected in our cohort. On the other hand, HPV45 (*HPV18* species)⁷⁶ was the second most common HPV subtype detected in samples of Croatian women. Due to their comparative high prevalence, HPVs 52, 45 and 58 need special consideration regarding the eventual cross-protection by HPV-vaccines as proven for HPV31, 33 and 45.⁷⁷ On the other hand, and based on the fact that E6/E7 oncoproteins of high-risk HPVs can convert noninvasive and non-metastatic breast cancer cells into invasive and metastatic ones,⁷⁸ we believe that more studies with a larger number of samples is necessary to determine the association between the presence of HPVs and tumor phenotype.

Likewise, in human breast cancer, the presence of EBV is detected in 30–50% of breast cancer cases, including TNBC worldwide (Table 3b).^{79–83} Our study indicated the individual presence of EBNA1, EBNA2, and LMP1 of EBV in 55/70 (78%), 31/70 (44%) and 0/70 (0%) of the TNBC cases, respectively. A probable explanation for the discrepant expression of the EBV variants can be that EBV infects rare tumor cells after neoplastic transformation has occurred. In addition, when latent state infection switches to lytic state infection, EBV-infected cells may be negative for EBERs. Previously, discrepancies in EBV

variants were reported in breast cancer samples^{84–89} and can be explained by the diverse roles of *EBNA* and *LMP* genes. During latent phases of EBV, there is different expression of the genes. While, during latency 0, no EBNA protein is expressed, during latency I and II, only EBNA1 is expressed. However, during latency III, all six EBNAs are expressed.^{90,91} Furthermore, the absence of LMP1 and the co-presence of both EBNA1 and EBNA2 in our cohort can be explained by the fact that in EBV-infected cells, in the latent phase, EBNA and LMP1 are expressed in a cancer-specific manner.⁹² While, in Burkitt lymphomas, EBNA and EBERs (controlled pattern of viral gene expression: latency 1) is expressed, in Hodgkin lymphomas, diffused large B cell lymphomas and EBV-positive gastric and nasopharyngeal carcinomas, all 10 EBV latent gene products (uncontrolled pattern of viral gene expression: latency II and III) are expressed.⁹² Regardless of the latency pattern, virtually all EBV-associated malignancies express EBNA and EBERs.⁹² Moreover, since PCR is a sensitive technique, the absence of LMP1 in all samples in our opinion is the result of the discrepant expression of EBV gene products in our cohort and not due to lytic state infection.

A previous IHC study reported EBV prevalence of 43% in all breast cancer cases, of which 49% were triple-negative.⁹³ Quantitative PCR analysis showed EBV positivity in 32.5% of breast cancer cases in French women; however, EBV-positive cases were more prevalent in TNBC (21%).⁹⁴ Additionally, an Algerian study reported EBV presence in 24% of the examined TNBC cases,⁹⁵ while in Qatar, EBV prevalence was detected in 40% of TNBC samples.³⁶ On the other hand, in Eritrea, PCR analysis revealed EBV in 28% of breast cancer cases.⁹⁶ We herein report that EBV is present in approximately 36% of TNBC cases in Croatian women. Therefore, our data indicate that the prevalence of EBV in TNBC tissues in Croatia is similar to the incidence present worldwide, including in the European region.

On the other hand, although, MMTV-like virus gene was detected in human breast cancer in varying proportions worldwide, ranging from 0% to 74% of cases⁴¹ (Table 4), studies analyzing the role of MMTV-like virus gene in TNBC samples are rare.

In the present study, we found that the MMTV-like virus gene is present in only 7% of TNBC samples from the Croatian cohort, similar to findings from a Brazilian TNBC cohort (4%).⁹⁸ Moreover, MMTV-like virus was detected in 19%, 17%, and 14% of breast cancer samples in Brazilian,⁹⁸

Chinese,¹⁰¹ and Tunisian¹⁰⁰ populations. Another study by Liu et al.¹⁰⁸ isolated a complete provirus from human breast cancer samples, while a study by Lawson et al.⁴⁵ detected MMTV-like antigens and sequences in benign lesions that developed to breast cancer in women during later years. MMTV-like virus gene was detected in 38% of human breast cancers but not in normal breast tissues.¹⁰⁹ A similar prevalence rate was reported by Ford et al. in breast cancer samples from Australia and Vietnam.¹⁰³ Etkind et al. performed PCR analysis using stringent hybridization techniques and detected MMTV-like virus gene in 37% of the human breast tumors.¹⁰⁵ However, other studies detected a higher presence of MMTV-like virus in breast cancer. In Morocco, the MMTV-like virus gene was detected in 57% of all breast cancer subtypes with no specific association with hormonal status.⁹⁹ Another investigation from Egypt by Loutfy et al.¹¹⁰ detected a higher prevalence rate of MMTV-like virus in both sporadic and germline breast cancer cases. In sporadic breast cancer, MMTV-like virus was present in 76% of the cases, while, 70% of hereditary breast cancer samples were positive for this oncovirus.¹¹⁰ However, a study in China found contrasting prevalence of MMTV-like virus in breast cancer samples from Northern and Southern China; while MMTV-like virus was present in 23% of the North Chinese women with breast cancer, in South Chinese women MMTV-like virus was prevalent in only 6% of the cases.⁹⁷ Furthermore, the study demonstrated the discrepant MMTV-like virus prevalence to be dependent on the worldwide distribution of *M. domesticus*, *M. musculus* and *M. Castaneus* mice; thus indicating the presence of house mice as a vital underlying environmental factor elucidating the geographical variation in the prevalence of human breast cancer.⁹⁷ On the contrary, studies failed to detect MMTV-like sequences in tumor tissue samples.^{111,112} These discrepancies can be attributed to technical, methodological, or epidemiological variations among studies.⁴¹

Intriguingly, based on previous works, including ours, high-risk HPVs and EBV co-presence can play an essential role in the onset and progression of different cancers, including oral, colorectal, cervical, and breast.^{22,42–44,113,114} In concordance, in our present investigation, we report that 16% of TNBC cases from Croatian women are co-infected with both HPVs and EBV; the presence of HPVs and EBV has been considered as a risk factor for TNBC.¹¹⁵ Moreover, in this study, we found that the co-presence of the three oncoviruses (HPV, EBV, and MMT-like virus) in only 3% of our TNBC cohort. However, similar to other studies, in this study, we did not find any association between the presence/co-presence of HPVs, EBV, and MMTV-like virus and clinicopathological characteristics.^{94,116} However, de Sousa Pereira et al. found a correlation between MMTV-like virus and clinicopathological features in Luminal B and HER2-positive samples,⁹⁸ thus indicating that MMTV-like presence may display subtype-specific effects in breast cancer. Thus, further studies are needed to evaluate the role of the MMTV-like virus in a larger breast cancer cohort.

In our previous studies, we have demonstrated that oncoproteins of HPVs (E5 and E6/E7) and EBV (LMP1 and/or EBNA1) can interact and cooperate in the initiation and/or progression of human oral and breast carcinomas *via* the EMT event;¹⁷ thus indicating a similar mechanism in the

Table 4. Prevalence of MMTV-like virus in breast cancer in different populations.

Country	Year	MMTV-like virus (%)	Reference
China	2021	21/119 (18%)	97
Brazil	2020	41/217 (19%) (Tissue samples) 17/32 (53%) (Blood samples)	98
Morocco	2014	24/42 (57%)	99
United Kingdom	2012	39/50 (78%)	79
Tunisia	2008	17/122 (14%)	100
China	2006	22/131 (17%)	101
Tunisia	2004	28/38 (74%)	102
Australia	2003	19/45 (42%)	103
Vietnam	2003	1/120 (1%)	103
Argentina	2002	23/74 (31%)	104
USA	2000	27/73 (31%)	105
Italy	1999	26/69 (38%)	106
USA	1995	121/314 (39%) (660-bp) 60/151 (40%) (250-bp)	107

pathogenesis of human breast malignancy. Moreover, several studies have pointed out the plausible role of MMTV-like virus in the onset and progression of breast cancer by stimulating IFN signaling and APOBEC-mediated mutagenesis in breast cancer, thus, allowing genomic instability and tumor development.^{117–119} Based on the role of HPVs, EBV, and MMTV-like virus in the pathogenesis of breast cancer, we suggest that oncoproteins of HPVs can interact with those of EBV (LMP1 and/or EBNA1) as well as MMTV-like virus resulting in the onset and progression of different types of cancers including breast.^{25,44,51}

The current study has several limitations. Firstly, we lacked an appropriate control group for the study (e.g., reduction mammoplasty samples); however, using the same methodology, we recently reported the prevalence of high-risk HPVs and EBV in breast cancer samples from Lebanon.⁴⁹ Secondly, since we did not have complete access to TNBC tissue samples, we could apply only one method (PCR-based) for HPV, EBV, and MMTV-like virus assessment. Of note, our previous studies exploring HPV and EBV in different types of cancers using PCR and IHC yielded comparable results.^{36,43,44,49,51}

Conclusions

Although the sample size used in the current study is relatively small, it indicates the presence/co-presence of the three oncoviruses in very few cases without any correlation with the studied clinicopathological parameters. Thus, we believe that future investigations with a larger cohort from different geographic locations are needed to confirm the co-presence of these oncoviruses in breast cancer to elucidate the exact role of their cooperation in breast carcinogenesis.

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