ORIGINAL ARTICLE

The prevalence of varicella zoster virus, herpes simplex virus type 2, and human papillomavirus in breast cancerous tissues and their adjacent ones in Iran

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Background: Breast cancer is the second type of cancer in the world. Some internal and external risk factors, especially infection diseases, can progress breast cancer. As the relation between varicella zoster virus (VZV), human papillomavirus (HPV), herpes simplex virus type 2 (HSV-2), and breast cancer has not been understood, it was attempting to find the effect of these viruses and breast cancer in this study. **Materials and Methods:** We collected 40 breast cancer and 50 healthy adjacent tissues from Taleghani and Imam Hossein Hospital, Tehran, Iran, in 3 years starting in 2017. After extracting DNA from breast tissues, multiplex polymerase chain reaction (PCR), nested PCR, and PCR were done to analyze the prevalence of HSV-2, VZV, and HPV. **Results:** Our results showed that HPV may be one of the important causes of breast cancer. Nested PCR illustrated nine breast cancerous tissues (mean age: 43) and three healthy adjacent ones (mean age: 41) were infected by HPV. Phylogenetic analysis illustrated that all of the infected HPV cancerous and healthy tissues were HPV 18 (except two healthy samples infected with HPV 6). Nevertheless, there were not any infected tissues by HSV-2 and VZV. **Conclusion:** It seems that HPV virus type 18 can have high prevalence in breast cancerous tissues in comparison with healthy adjacent ones, and it is likely to have an effect on breast cancer progression. However, the opposite trend is true for HSV-2 and VZV as we did not find any differences between different kinds of breast tissues.

Key words: Herpes simplex virus type 2, human papillomavirus, varicella zoster virus

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INTRODUCTION

Statistics have shown that the second type of malignant tumor in all around the world is breast cancer,[1,2] and it has remained as a challenging issue for clinicians to recognize this health problem by different biomarkers. Genetic mutation, heterogeneity, complex etiology, and the various clinical manifestations can have deteriorating effects on the progression of breast cancer. Therefore, numerous external and internal risk factors could progress the pathogenesis of breast cancer. External risk factors, including obesity, alcohol consumption, smoking, and the level of melatonin hormone, and internal risk factors; such as epigenetic and genetic, could trigger signaling pathways in breast cancerous cells through the deregulation of a number of genes.[3-6]

There are concrete relations between different types of cancers and inflammatory diseases. Take, for example, the relation between gastric cancer and *Helicobacter pylori*.[7,8] Indeed, the effect of different infectious diseases, especially viral ones, on some types of cancers cannot be ignored.[9,10] It is confirmed that human papillomavirus (HPV), for instance, can progress cervical cancer detrimentally.[11] Moreover, HPV may have the ability to trigger the deterioration of breast

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cancer.[12] This virus belongs to human papilloma viridea, having double-stranded DNA, which can express some oncogenes such as E7 and E6 that help this virus to escape from the immune system.[13] E7 can divide the Rb and E2F from each other; therefore, cells are induced to express more proteins. With disrupting P53 (tumor suppressor protein) through ubiquitination, E6 is able to decrease the apoptosis rate.[13,14] Among different types of HPV, HPV-16 and-18 have the most ability to develop human cancer progression.[15]

Besides, Herpesviridae infections have the potential to transform cells, and it is the reason why this virus family results in different kinds of cancers, including Hodgkin's lymphoma, Burkitt's lymphoma, Kaposi sarcoma, nasopharyngeal, and stomach carcinoma.[16] Not only can this virus family cause the chromosomal mutation, but it is also able to affect on the overexpression of preexisting oncogenes and gene amplification in neoplastic tissue.[16] In terms of clinical presentation and signs of these viruses, human Herpesviridae is classified into some subgroups, ranging from alpha-, beta-, to gamma-herpesviruses subgroups stimulating numerous health problems.[17-19] To illustrate, 84 different structural and nonstructural proteins can be up or downexpressed by alpha subgroups, which may impress the signaling of cancer progression.[20] Furthermore, BamHI A Rightward Frame 0 (BARF0) , EpsteinBarr nuclear antigen 1 (EBNA1), and EBERs (Epstein-Barr virus-encoded small RNAs) expression are likely to suppress hsa-miR-200a and 200b which are tumor suppreors ; therefore, decrease in the expression of Ecadherin in different human cancer tissues and cancer progression can be observed.[21]

With available technology, the relation between HPV, Herpesviridae, such as herpes simplex virus (HSV)-2 and varicella zoster virus (VZV), and breast cancer has not been clear. In this study, we endeavored to detect these viruses and identify the prevalence of HPV and Herpesviridae in breast cancer, in Iran.

MATERIALS AND METHODS

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Sample

In this case–control study, 40 breast cancer and 50 healthy adjacent tissues were collected from Taleghani and Imam Hossein Hospital, Tehran, Iran, in 3 years starting in 2017 (All of our patients were female). To confirm the stage of all tissues, three pathologists analyzed the tissues' stages. Table 1 indicates all recorded personal information.

Table 1: 40 sample's characteristics of patients suffering from breast cancer

Samples characteristics of patients	Cases (%)
≥60 years old	28 (70)
Tumor localization	
Left	18 (45)
Right	22 (55)
Family history	
Absent	34 (85)
Present	6 (15)
Lymph node metastasis	
Negative	18 (45)
Positive	22 (55)
Tumor size (cm)	
>2	24 (60)
<2	16 (40)
Tumor stage	
I–II	20 (50)
III–IV	20 (50)
The status of ER	
Negative	22 (55)
Positive	18 (45)
The status of PR	
Negative	18 (45)
Positive	22 (55)

ER=Estrogen receptor; PR=Progesterone receptor

DNA extraction

To detect the HPV, VZV, HSV-2, and cytomegalovirus (CMV) in breast cancer tissues, purified DNA was required. We extracted DNA by digesting all tissues (incubating all of them in 37°C in a digesting buffer containing proteinase K, overnight). Then, proteins were discharged in three steps by phenol, phenol–chloroform, and chloroform. (All tissues were kept in 37°C overnight by the digesting buffer containing proteinase K. After that, the same amount of phenol [CinnaClon Co., Iran] and tissues were mixed. Then, the same procedure was employed for two other processes with phenol–chloroform and chloroform to discharge protein).

B-globin gene polymerase chain reaction

The presence of human β-globin gene (100 bp), in every tissue, confirmed the correct DNA extraction procedure. In this study, 1 µl forward and reverse primer (10 pmol the final amount of the primer in the reaction was used in this study), 12.5 µl master mix, 8.5 µl sterile water, and 1 µl DNA in final 25 µl were combined, and all samples in 5 min in 95°C as first denaturation, 30 cycles of 95°C for 30s, 55°C for 30s, 72°C for 30s, and 72°C for 7 min were incubated. Every negative sample was extracted again and double-checked.

Nested polymerase chain reaction

With two scheduled programs in nested PCR, we detected HPV in breast cancer tissues. First of all, we combined 12.5 µl master mix PCR, 2.5 µl DNA, 2.5 µl forward primer (MY-9) and reverse primer (MY-11) [Table 2], and 2.5 µl sterile water. The first PCR schedule program was 5 min initial denaturation at 94°C, 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 7 min. Then, 25 µl master mix, 2.5 µl forward primer (GP5), 2.5 µl reverse primer (GP6), 15 µl sterile water, and 5 µl first PCR products were combined. The schedule template for the second step was 4 min initial denaturation at 94°C, 40 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 2 min, extension at 72°C for 2 min, and final extension at 72°C for 4 min. The final PCR product, which we detected by running on electrophoresis PCR, was 150 bp. We employed sterile water and HeLa cell line DNA as negative and positive controls, respectively.

Sequencing

Gp5 forward primer of nested-PCR was employed to sequence. After purifying, the PCR products were sequenced on forward direction inside an ABI PRISM 310 genetic analyzer (PE Applied BioSystems Inc., Foster City, CA, USA) using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied BioSystems Inc.).

Phylogenetic analysis

MEGA software version 7.0 (Biodesign Institute, Tempe, AZ,) aligns reference sequences from the GenBank database with the results of L1 HPV protein sequences. Neighbor joining was utilized in the mentioned software to analyze phylogenetics. In the GenBank database, we submitted all the results.

Multiplex polymerase chain reaction and polymerase chain reaction

Twenty-five microliter master mix, 0.5 µl forward and 0.5 µl reverse primers of HSV-2, VZV (1.5 µl forward primer and 1.5 µl reverse primer), 4 µl DNA template, and 18 µl sterile distilled water in final 50 µl were mixed. Moreover, we set up the PCR to detect CMV. We mixed 12.5 µl master mix, 4 µl DNA templates, 6.5 µl sterile distilled water, and 1 µl forward and 1 µl reversed primers.

Furthermore, we set up a multiplex PCR to detect VZV and HSV-2. In this kind of PCR, we combined 25 µl master mix, 18 µl sterile distilled water, 4 µl DNA template, and 0.5 µl forward and 0.5 µl reverses primers of HSV-2, VZV (1.5 µl forward primer and 1.5 µl reverse primer) in final 50 µl.

The PCR schedule program to detect CMV, HSV-2, and VZV was an initial denaturation step at 95°C for 2 min, followed by 40 cycles at 95°C for 2 s, 58°C for 15 s, and 72°C for 15 s, with a final extension at 40°C for 30 s. Gel electrophoresis indicated the positive samples (UL55 gene encodes glycoprotein B (gB) were employed as positive control for CMV and glycoprotein G (gG) were cloned in the plasmid for HSV-2 and VZV positive controls).

Table 2: Nucleotide sequences of primers used for nested polymerase chain reaction and multiplex polymerase chain reaction

Name of primers	Sequence of primers
PCR-HSV2-F	5'-TATGCCTATCCCCGGTTGGA-3'
PCR-HSV2-R	5'-CGTGCCATCCGAATAAACGTG-3'
PCR-VZV-F	5'-TTGTGTCGGTCTCTCCAAGC-3'
PCR-VZV-R	5'-TACGTCTTCAACCTCACGCC-3'
MY-9	5'-CGTCC(A/C)A(A/G)(A/G)GGA(A/T)ACTGATC-3'
MY-11	5'-GC(A/C)CAGGG(A/T)CTATAA(C/T)AATGG-3'
GP-5	5'-TTTGTTACTGTGGTAGATACTAC-3'
GP-6	5'-AAAAATAAACTGTAAATCATATTC-3'
GAPDH F	5'-ATGTTCGTCATGGGTGTGAA-3'
GAPDH R	5'-GGTGCTAAGCAGTTGGTGGT-3'

PCR=Polymerase chain reaction

Statistical analysis

SPSS 20.0 software (IBM, Armonk, NY) was used to find the mean age of cancerous samples and healthy ones. Furthermore, the Chi-square test was used to find the associations and comparisons of proportions using Prism version 6. Statistical significance was set at 0.05.[22]

RESULTS

To detect HPV, HSV-2, and VZV, we collected 40 breast cancer and 50 healthy adjacent tissues. PCR, nested PCR, and multiplex PCR were employed in different schedule temperature programs. Two expert pathologists analyzed all tested tissues, and it was confirmed that more than 50% of patients suffered from a tumor with stage III-IV [Table 1]. β-globin gene was used as an internal control, and every negative one was double-checked. After extracting the DNA of tissues, nested PCR showed 9 breast cancerous tissues (mean age: 43) and 3 healthy ones (mean age: 41)

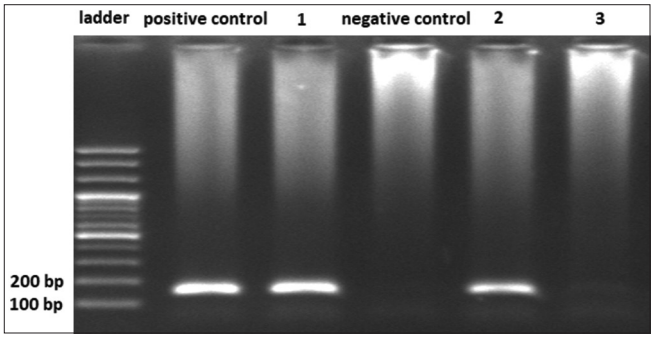

Figure 1: Human papillomavirus (HPV) (150 bp) in nested polymerase chain reaction (PCR). As depicted, these samples are positive for HPV infection. Positive and negative controls are used to confirm the accuracy of the PCR procedure

were infected with HPV [Figure 1] (9 cancerous tissues with HPV-18, 1 normal adjacent sample with Hpv-18, and 2 other normal adjacent tissues HPV-6); nevertheless, there were not any infected tissues by HSV-2 and VZV.

After determining that HPV can have a crucial effect on breast cancer progression, we investigated whether any specific type of HPV has the most prevalence. The sequences indicated that all infected HPV cancerous and healthy tissues are HPV 18 except two samples which involved HPV 6 in healthy tissues. The phylogenetic characteristic of the HPV virus is shown in Figure 2. Furthermore, cancer-involved samples illustrated more HPV positive in comparison with adjacent tissues in Table 3 ($P = 0.02$).

DISCUSSION

According to previous studies, different risk factors, including age, sex, diet high in salt and fat, alcohol consumption, smoking the level of melatonin hormone, and alteration in gene expression, can cause breast cancer progression. The catch is that infectious risk factors may be supposed to accelerate breast cancer invasion. In 2012, it was estimated more than 1.7 million women were suffering from breast cancer diseases.[23] Breast cancer also is responsible for about 14.3% of annual mortality worldwide.[24] The diagnosis of different studies has shown, breast cancer comprises 23% of all female cancers.[25] As the effect of HPV

Table 3: The comparison of cancer-involved specimens to normal adjacent breast tissues concerning human papillomavirus-DNA positivity

	HPV (+), *n* (%)	HPV (–), *n* (%)	Total, *n* (%)
Cancer involved specimen	9 (22.5)	31 (77.5)	40 (100)
Normal adjacent tissue	3 (6)	47 (94)	50 (100)
P	0.0297		

HPV=Human papillomavirus

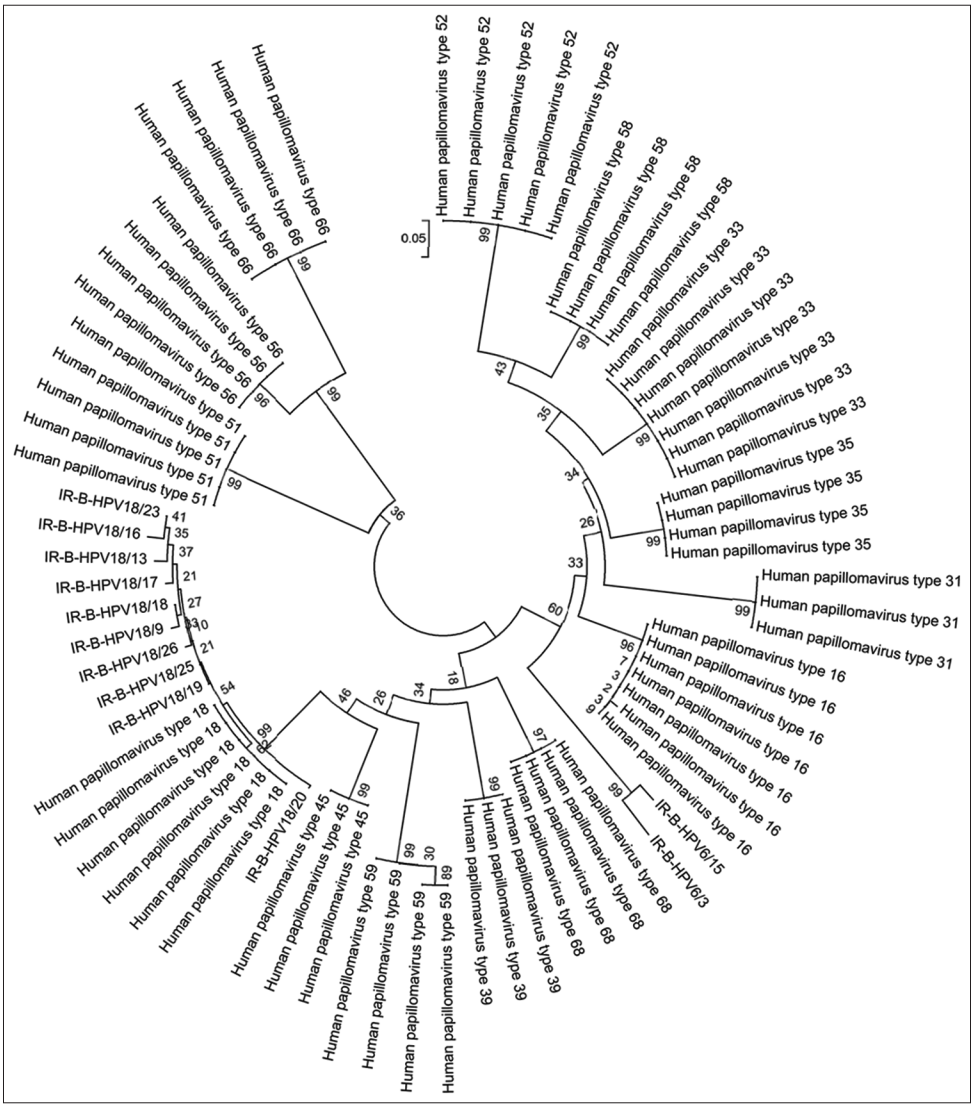

Figure 2: The phylogenic result of human papillomavirus-positive sample. HPV: Human papilloma virus

and some members of herpetic infection on breast cancer, in Iran, are not clear, we detected these viruses in the biopsy of patients suffering from this type of cancer.

Our results indicated the number of cancerous tissues ($n = 9$), infected by HPV, is higher than healthy tissues ($n = 3$), and HPV18 was observed in 1 healthy and 9 cancerous tissues and HPV6 infected 2 healthy samples. These results are similar to some other studies, for example, Niloofar Khodabandehlou showed 48.6% of cancerous tissues and 16.1% of healthy ones were positive for HPV, and most genotypes of samples were HPV18.[26] In northeast Brazil, 49.5% and 15.8% of breast carcinoma samples and normal adjacent ones were infected by HPV, respectively. About 15.2% of samples indicated HPV 6 and 11 by *in situ* hybridization with biotin-labeled probes and PCR,[27] and HPV16 was detected in 29.4% of breast tumorous tissues.[28] Another study indicated 44.4% of 273 Italian women were HPV positive, and it was supposed that HPV can increase breast cancer progression.[29] Sigaroodi *et al*. showed 25.9% of samples of breast cancer tissues presented the HPV DNA in comparison with 2.4% of women with noncancer status ($P = 0.002$), and 53.34% of HPV DNA were HPV16,18, but the others were HPV-6, HPV-11, HPV-23, HPV-15, and HPV-124.[30] On the other hand, some studies claimed there is no relation between HPV and breast cancer. Kazemi Aghdam *et al*. showed in 75 normal breast tissues and 75 paraffin-embedded breast cancer tissues, there are no HPV-positive samples.[31] Furthermore, in 2 of benign conditions and 76 carcinomas, HPV was not found.[32] Sara Bønløkke found the comparison of HPV in case and control samples was not meaningful (2.15 vs. 1.00%, $P = 0.61$ and 1.08 vs. 0.00%, $P = 0.48$).[33] Furthermore, in 70 malignant breast tumors (cases) and 70 controls, there is no association between HPV and breast cancer.[34]

In this study, two normal adjacent breast tissues presented the HSV-1 DNA, which may demonstrate this virus can have the potential of killing breast cancer cells. As HSV-1 has special genes that may impact on patients suffering from breast cancer, different studies investigated to shed light on this issue. Kuruppu D suggested that HSV-1 can be employed as a novel way to treat breast cancer metastases.[35] Observations have shown the HSV type 1 mutant HF 10 is able to cause a proportional reduction in the size of breast tumor tissues.[36] To eradicate breast cancer, it was published to use oncolytic HSV1 following doxorubicin treatment.[37] The third-generation oncolytic HSV (oHSV) vector G47Δ can kill more than 98% of breast cancer stem cells.[38] On the other hand, some studies bring a conflict on this issue and claimed with oncogenes, HSV-1 can be considered a cofactor in breast cancer, for example, the presence of HSV-1 in 31.8% (7 out of 22) showed the HSV-1 has a concrete relation with breast cancer.[39]

VZV is one of the herpetic viruses that we investigated the breast cancer prevalence. In this study, we did not find any VZV neither in breast cancer tissues nor normal adjacent ones (There were not any VZV infections in their tissues). Eghbali *et al*. found no meaningful association of this virus and breast cancer.[40] As VZV is able to be activated in stress situations, in most chemotherapy and radiotherapy, there is an increased risk in VZV infection. After radiotherapy in the study of Lai YL, there was a higher risk of VZV infection 1.51-fold (95% confidence interval = 1.06–5.16, $P = 0.02$, IRD = 4.98/10000 person-years); moreover, in patients received radiotherapy who aged >65 years, the risk of VZV was 3.85-fold higher.[41] 1.8–8.4-fold higher risk of a VZV was observed in patients suffering from renal failure, diabetes mellitus, and malignancies.[42] Fourteen patients (15.2%), during systemic chemotherapy in solid breast cancer, showed VZV infection diseases.[43] In more than 56% of patients received radiotherapy plus combination chemotherapy. (This study has a limitation, including the low numbers of samples, and lack of sample characteristics of the control group).

CONCLUSION

It seems that HPV virus type 18 can have high prevalence in breast cancerous cells in comparison with healthy ones, and it is likely to have an effect on breast cancer progression. However, the opposite trend is true for HSV-2 and VZV as we did not find any differences between different kinds of breast tissues.

Ethics approval and consent to participate

This study has been conducted in the Department of the School of Medicine at Shahid Beheshti University of Medical.

Consent for publication

Written informed consent for publication was obtained from the participants or their guardians.

Availability of data and materials

Please contact the author for data requests.

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Conflicts of interest

There are no conflicts of interest.

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