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High Frequency of HPV Genotypes 16 and 18 Found in Breast Cancer Patients: Evidence for a More Comprehensive HPV Vaccination Program in Iran

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Abstract

Background & Objectives: Papillomaviruses are found in many different types of infections and in a wide range of animals and humans. They can cause health problems, including benign and malignant tumors. In the present study, the association between human papillomavirus (HPV) infection and breast cancer (BC) in Iran was investigated.

Materials & Methods: In this cross-sectional study, the presence of the HPV genome was investigated in BC-suspected tissues for the first time in Qom Province, Iran. A total of 400 samples (including 200 BC-suspected tissue samples and 200 blood samples of women without BC) were collected from women referred to two cancer-specific general hospitals. To determine the presence of the L1 gene of HPV in the collected samples, nested polymerase chain reaction (PCR) was performed. Then, HPV-positive samples were tested by PCR using high-risk specific HPV-16 and 18 primers.

Results: Out of 200 BC-suspected tissue samples, 172 were malignant (in terms of pathology). Based on the nested PCR method, the L1 gene of HPV was detected in 12% (24/200) of the BC-suspected tissue samples and in 1.5% (3/200) of the blood samples from women without BC. The high-risk HPV genotypes (which were the predominant types) were present in 75% of the samples.

Conclusion: The results of the current study show a high frequency of HPV-16 and 18 genotypes in human BC in Iranian women. This is almost certainly due to poor rates of HPV vaccination, and it is strongly recommended that health organizations (such as the World Health Organization [WHO]) ensure adequate coverage of highly effective HPV vaccination in Iran.

Keywords: Human Papillomavirus; Breast cancer; Nested PCR.

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Introduction

Cancer is an abnormal proliferation of cells with the potential to invade or spread to other parts of the body (1). Breast cancer (BC) is one of the most common cancers worldwide and a leading cause of mortality in women (2). According to data from the World Health Organization, 2, 26, 419 new breast cancer cases were reported worldwide in 2020 (3). In the United States of America, more than 1,000,000 new patients are diagnosed each year (4). In Iran, it is the most common malignancy in women (5). The incidence of BC is increasing everywhere due to increases in its risk factors (6). Several risk factors such as age, hormones,

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alcohol consumption, diet, cigarette smoking, family history and oncogenic viruses are associated with BC. The most suspected viruses in BC are hepatitis B and C viruses (HBV and HCV), human papillomavirus (HPV), Epstein-Barr virus (EBV), human T-cell leukemia virus type 1 and 2 (HTLV-1 and 2), human herpes virus 8 (HHV-8), mouse mammary tumor virus (MMTV), and, more recently, bovine leukemia virus (BLV)(2, 7).

HPV infections are usually transmitted sexually. HPV are very diverse and only infect humans. They infect epithelial cells of the skin and mucous membranes and produce hyperplasias, such as warts (8, 9). Characteristically, HPV has been implicated in some cases of head and neck cancer, anogenital tumors, cervical cancer, and BC. More than 100 HPV types have now been identified, 10 of which are classified as high-risk types because they lead to abnormal cell changes that can cause cancer. High-risk HPV types 16, 18, 31, 33, 35, and 45 have been formally recognized as dominant in 70% of all genital or cervical cancer samples. Previous studies have demonstrated the presence of high-risk HPV types 16, 18, and 33 in BC samples (7, 10).

HPV belongs to the Papillomaviridae family, which has a circular, double-stranded DNA genome with a size of approximately 8 kilobases (kb). Despite its small size, its molecular biology is very complex. Viral proteins include 3 oncogenes, E5, E6, and E7 (as stimulators of host cell proliferation), 2 regulatory proteins, E1 and E2 (modulate transcription and replication), and 2 structural proteins, L1 and L2 (form the viral capsid). The open reading frames of E1, E2, L1, and L2 are particularly well conserved among all members of the family (11, 12). The E7 protein interacts with retinoblastoma (RB), resulting in the release of E2F, a transcription factor that promotes cell proliferation. E7 upregulates the S-phase genes, cyclin A and cyclin E, but in contrast it inhibits cyclin-dependent kinase inhibitors, such as cyclin-dependent kinase inhibitor (WAF -1; known as p21) and kinesin-like protein (KIP -1; known as p27)(13).

Nested polymerase chain reaction (PCR) is a modification of PCR and a modern molecular method to improve sensitivity and specificity. This method uses two sets of primers and t consecutive PCR reactions (14).

In a previous study (2019), the authors showed that BLV DNA is present in some human BC samples (15); accordingly, the aim of the current study was to investigate the presence of HPV in women's breast tissue and blood samples using the nested PCR method in Qom Province, Iran.

Materials and Methods

Samples Collection

In this cross-sectional study, a total of 400 samples were collected from women referred to 2 general hospitals with special departments for cancer patients in Qom province, Iran, from July 2019 to October 2020. A total of 200 BC-suspected tissue samples were evaluated to determine a possible association between HPV infection and the manifestations and progression of BC. The sample size required for the study was calculated according to the desired absolute precision of 95% and an expected prevalence of 25% using the following Cochran formula.

Tissue samples were formalin-fixed and obtained from women who had undergone breast surgery (mastectomy and/or mammoplasty). Mammoplasty samples were assumed to be normal breast tissues classified as non-tumor and control samples. In addition, 200 blood samples were randomly collected from 25-70 year-old women without BC. The donor population of the blood samples was completely different from that of the breast tissue samples.

Preparation of Samples

Using the Bioneer kit (South Korea) and according to the manufacturer's instructions, 25 to 50 mg of tissue samples were placed in 1.5 mL tubes, and 200 µL of tissue lysis buffer

(TL buffer) was added to each tube. Then, the samples were ground with sterile distilled water and disposable pestles under liquid nitrogen. In addition, EDTA-blood tubes (200 µL) were used for blood collection, and finally, the samples were stored at -20 °C until analysis.

DNA Extraction and PCR

The presence of HPV DNA in the breast tissue and blood samples was analyzed using the nested PCR method. DNA was extracted using a genomic DNA extraction kit (Bioneer, South Korea) according to the manufacturer's instructions. The primers used for PCR were designated based on the L1 gene of HPV (GenRunner and CLC Sequence Viewer 6 software packages). The specificity of the primers for HPV and their sequence alignments were confirmed using the NCBI BLAST program. Then, they were synthesized by CinnaGen Company in Iran (Table 1). GAPDH gene fragments were amplified as controls to confirm the quality of extracted DNA from the samples. The HeLa cell line (ATCC®CCL-2) and DNA extracted from the blood serum of HPV-negative samples were used as positive and negative controls, respectively.

The second set of primers is within the sequence that the first set of primers amplifies in the nested PCR method. The amplification protocol of GAPDH and E7 genes is shown in Table 2. Also, HPV-positive samples were tested by the PCR method using high-risk specific HPV-16 and 18 primers.

For electrophoresis evaluation, 5 µL of each reaction mixture was visualized on a 1% agarose gel with a safe stain (GelRed®, Biotium Company, California) using a UV transilluminator.

Table 1. Sequences of the primers used for detection of HPV and its genotypes

Gene	Primer sequence	product size
L1	Outer primer MY09: 5′-CGTCC(A/C)A(A/G)(A/G)GGA(A/T)ACTGATC-3' MY11: 5′-GC(A/C)CAGGG(A/T)CTATAA(C/T)AATGG-3' Inner primer GP5+ :5′-TTTGTTACTGTGGTAGATACTAC-3′ GP6+: 5′-AAATCATATTCCTCAACAT¬GTC-3′	150 bp
E7 (HPV-16)	Forward: 5′-TTATGAGCAATTAAATGACAGCTCAG-3′ Reverse: 5-TGAGAACAGATGGGGCACACAAT-3′	215 bp
E7 (HPV-18)	Forward: 5′-GACCTTCTATGTCACGAGCAATTA-3′ Reverse: 5′-TGCACACCACGGACACACAAAG-3′	236 bp
GAPDH	Forward: 5′-GCTCGGTGCCTTTAGTGATGG-3′ Reverse: 5′-CGATCCTGAGACTTCCACACTG-3′	255 bp

Table 2. The protocol for the first and second rounds in nested PCR reaction

Gene	-	Initial denaturation	Subsequent denaturation	Annealing	Extension	Final extension
L1	First round	94°C 5min 1x	94°C 1min 34x	53°C 30s 34x	72°C 30s 34x	72°C 5min 1x
	Second round	94°C 5min 1x	94°C 1min 34x	55°C 30s 34x	72°C 45s 34x	72°C 5min 1x
E7	-	94°C 5min 1x	94°C 30s 30x	58°C 30s 30x	72°C 40s 30x	72°C 5min 1x
GAPDH	-	94°C 8min 1x	94°C 1min 35x	64°C 1min 35x	72°C 1min 35x	72°C 7min 1x

Statistical Analysis

Data were analyzed using SPSS version 22.0 (SPSS Inc., Chicago, Ill., USA) and Microsoft Excel software. The t-test was performed, and differences were considered significant at $P < 0.05$.

Results

In total, 172 out of 200 BC-suspected tissue samples collected from women undergoing breast surgery were malignant (in terms of pathologic diagnosis). Other samples were reported as non-malignant and non-tumor samples (tissue samples taken from mammoplasties and/or after chemotherapy).

Based on the nested PCR method, the L1 gene of HPV was detected in 12% ($P < 0.05$) of BC-suspected tissue samples. The malignant samples of HPV-positive individuals were invasive ductal carcinoma (microscopic diagnosis) and grade III (histologic grading of cancer). The high-risk HPV genotypes (types 16 and 18) in samples were 18/24 (75%; $P < 0.05$). More details are shown in Table 3.

HPV was detected in 3/200 (1.5%; $P < 0.05$) of blood samples collected from women without BC in the age range 30-53. This report of virus present in the human blood cause facilitates the transmission of HPV infection to human.

The amplification results of L1 and E7 genes of HPV by the PCR method are illustrated in Figures 1 and 2, respectively.

It is notable that HPV DNA was identified even after chemotherapy in some BC samples.

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Table 3. Distribution of HPV in breast cancer-suspected tissue samples
Malignant and non-malignant tissue samples were obtained from women undergoing breast surgery for mastectomy. Mammoplasty specimens and some mastectomy specimens after chemotherapy were considered as non-tumor specimens. Mammoplasty samples never had BC and chemotherapy samples before surgery had a history of BCbut no current BC.

	breast tissue samples	L_1+ samples	L_1- samples	HPV-16	HPV-18
Number	200	24 (12%)	176 (88%)	7 (29.1%)	11 (45.8%)
Malignant	172 (86%)	20 (10%)	152 (76%)	7	11
Non-malignant	8 (4%)	0	8 (4%)	0	0
Non-tumor	20 (10%)	4 (2%)	16 (8%)	0	0
Donor age range	25-81	36-65	25-81	-	-

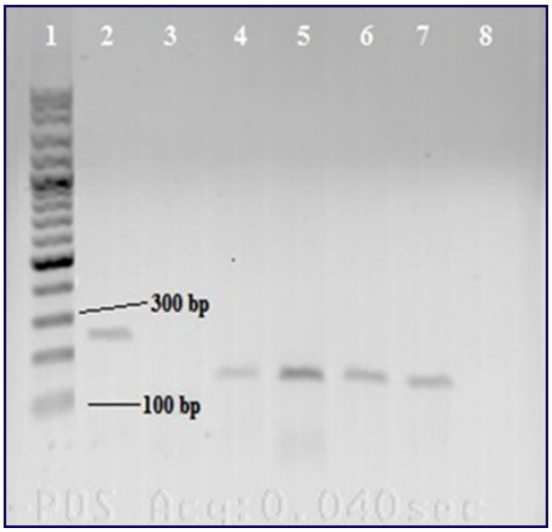

Figure 1. Amplification of L1 gene (for HPV detection) in some samples. Lane 1: 100 bp DNA ladder; Lane 2: 255 bp GAPDH gene; Lane 3: negative control; Lane 4: 150 bp positive control; Lane 5-7: 150 bp positive spcimen; Lane 8: negative spcimen

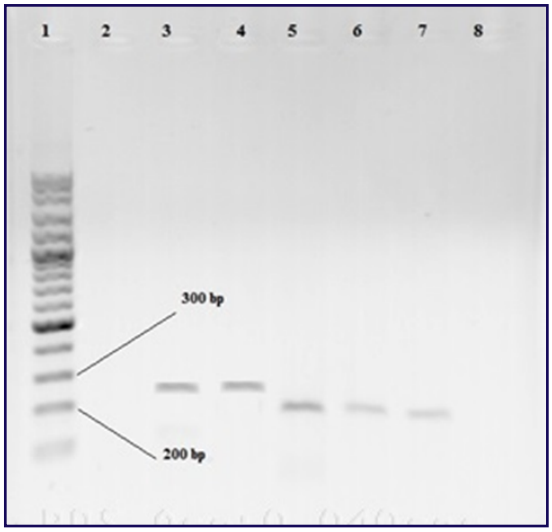

Figure 2. Amplification of E7 gene (for HPV genotypes 16 and 18 detection) in some samples. Lane 1: 100 bp DNA ladder; Lane 2: negative spcimen; Lane 3: 236 bp E7 gene (HPV-18) positive control; Lane 4: 236 bp E7 gene (HPV-18) positive spcimen; Lane 5: 215 bp E7 gene (HPV-16) positive control; Lane 6,7: 215 bp E7 gene (HPV-16) positive spcimen; Lane 8: negative control

Discussion

Cancer is the third common cause of death in Iran (16). Moreover, 10%-15% of cancers worldwide can be associated with viral infections (17). Despite the development of BC therapies such as surgery and chemotherapy, BC is still one of the major causes of death in women (18). Previous studies have demonstrated that there are oncogenic viruses in normal and malignant human breast tissues (7).

To our knowledge, this is the first epidemiological study on the presence of HPV in women with and without BC in Qom Province, Iran. Some researchers such as Gopalkrishna et al. in India (19), Gannon et al. in Australia (20), Vernet-Tomas et al. in Spain (21), and Karimi et al. in Iran (22) rejected the role of HPV in BC. On the other hand, most of the other researchers have reported the presence of HPV DNA in BC to be as high as 86% (7, 23). In the current study, the L1 gene of HPV was detected in 24/200 (12%) of BC-suspected tissue samples, while other studies showed that the frequency of HPV DNA in women with BC was 39%-50% in Australia (24, 25), 86% in Germany and the United States (26), 26% in Argentina (17), 21% in Japan (27), 51.8% in Spain (28), 18.1% in Pakistan (29), 49.5% in Brazil (12), 44.4% in Italy (30), 23.9% in Mexico (31), 25.9% in Mazandaran Province, Iran (6), 26.2% in Mashhad Province, Iran (32), 48.6% in Kashan Province, Iran (13), 11.8% in Tehran Province, Iran (33), and 33.8% in Tabriz Province, Iran (34). Our group has previously described the BLV presence in 38% of Iranian women with BC (15). We speculate that these differences should be more related to differences in DNA extraction methods, HPV detection methods, number of samples, and different prevalence. In the current study, HPV DNA was detected in blood samples collected from women without BC (1.5%). The prevalence of HPV DNA was 25% in patients with lymphocytic leukemia (16). Previously, it was shown that the BLV was present in 16.5% of Iranian women without BC (15). The presence of the virus in human blood is noticeable because it facilitates the transmission of viral infection to human and the spread in all organs. The oncogenic effect of HPV on breast tissue could be via the E6 and E7 oncogenes (21). Thus, for the sake of tumor suppressor gene suppression, cancer will be produced. The previous studies have demonstrated the presence of high-risk HPV types 16 and 18

in BC specimens. High-risk HPV DNA types 16 and 18 were detected in 53.34%, 66.6%, and 22.2% of BC samples by Sigaroodi et al., Ranjbar-Zeidabadi et al., and El-Sheikh et al., respectively (6, 10, 16). In the current study, high-risk HPV genotypes based on the E7 gene were the most prevalent genotype (75%), which is higher than several countries and is considered high frequency. Qom Province is a destination for many travelers and migrants, most of whom come from different provinces of Iran and various countries in Africa and Asia that are endemic with many diseases and weak healthcare systems. The prevalence of these HPV types is 21.4% in China (18), 64%-72% in Australia (35, 36), and 42% in Pakistan (29).

Moreover, we investigated the role of age in HPV-positive samples by comparing women with and without BC. The age range was 30-53 years in women without BC and 36-65 years in women with BC. These results suggest that symptoms of HPV infection occur at older ages, about 5-10 years later.

Maldonado-Rodríguez et al. concluded that virus presence is not a sufficient condition for developing cancer (31). However, this study, along with other studies, showed a high frequency of high-risk HPV genotypes infection in Iran. It is worth noting that due to economic problems and sanctions, HPV vaccination is so expensive in Iran that most people are unable to get vaccinated, unsafe sexual activity is reportedly increasing, resulting in an increased risk of sexually transmitted infections and an increased prevalence of HPV (32). Accordingly, these reports indicate that high-risk HPV genotypes infection will markedly increase in the next years. If health organizations are unable to reduce viral transmission by vaccination or regular screening of high-risk groups, we will experience higher rates of cancer in the future.

Conclusions

The results of the current study show an association between human BC and HPV infection in Iranian women. HPV cell culture is not possible; therefore, the nested PCR method is the best method for the detection of HPV. Accordingly, this method is recommended, along with the Pop smear test, for the detection of high-risk HPV. Also, the results indicate a high frequency of genotypes 16 and 18, indicating the need for further use of highly effective HPV vaccines. Therefore, it is strongly recommended that health organizations (such as the World Health Organization [WHO]), together with government measures, ensure adequate coverage of highly effective HPV vaccines against genotypes 16 and 18 in Iran.

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