Detection of Human Papillomavirus Genotypes in Breast Cancer Patients

Morvarid Golrokh Mofrad  
Razi Vaccine and Serum Research Institute

Zohreh Azita Sadigh  
Razi Vaccine and Serum Research Institute

Sanaz Ainechi  
University of Southern California

Ebrahim Faghihloo (faghihloo@gmail.com)  
Shaheed Beheshti University of Medical Sciences  https://orcid.org/0000-0002-8669-305X

Research

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Abstract

Background: The role of viruses as a cause of breast cancer (BC) have been significantly investigated in recent years. Human papillomavirus (HPV) has been detected in invasive breast carcinomas, while most studies have only focused on the detection of viral DNA, we aimed to determine genotypes of the culprit HPV's.

Methods: We collected and analyzed 70 Formalin Fixed Paraffin Embedded (FFPE) blocks including 59 BC samples, and 11 benign breast lesions as control from Iranian patients using nested PCR. Real-time PCR utilized as a confirming test to nested PCR findings. Genotyping of HPV positive samples was performed, the samples were also subjected to a multiplex PCR to detect herpes simplex-1 (HSV-1), herpes simplex-2 (HSV-2), varicella zoster virus (VZV) and cytomegalovirus (CMV) in BC.

Results: Papillomavirus DNA was present in 7 of 59 BC samples (11.8%); while none was detected in control samples. The most prevalent type was HPV18, followed by HPV 6. No HPV16 genotype was detected .All HPV positive patients had high tumor grades (II/ III) with histologic diagnosis of ductal carcinoma. The patient age range was 33 to 73 years with median of 51 years. Most of HPV positive patients had low levels of education. Also 5 of 59 BC specimens (8.47%), were positive for HSV-1.

Conclusions: Our results suggest a carcinogenesis role for High risk HPVs in breast tumors. Our findings of HPV6 and HSV1 genotypes in BC samples, propose a cancer causing role for low risk HPVs and herpes viruses. Further large scale studies are warranted to assess the significance of our findings.

Background

Breast Cancer (BC) is the most prevalent cancer worldwide among women. BC accounts for approximately 30% of female cancers and is the most frequent cause of death in women (1, 2). Over the last decade incidence rates of BC have raised by around 20% worldwide. In spite of the fact that the incidence rate of BC is higher in developed countries, has higher mortality in developing countries. With more than 55% of breast cancer related deaths occur in them (3, 4). Lack of early detection programs, inadequate diagnostic and treatment facilities are the main reasons for low survival rate in most developing countries (5, 6). The fifth most common cancer related death in Iran is BC .(7). Different factors, such as family history, genetics, decreased childbearing and breast feeding, harmful dietary, previous benign breast disease, radiation, oral contraceptives, smoking and alcohol intake are considered as risk factors in the onset of cancer (8-10). Meanwhile, a pathogenesis role for some infectious agents including viruses has been questioned. (11-14).

Some studies have shown the role of human papillomavirus (HPV) in different cancers (15-17). Over the past two decades various studies have suggested a role for HPV in breast cancer (18-22).

Thus far over 200 HPV types have been identified and most of them fall within three genera: alpha (α), beta (β) and gamma (γ). While alpha type (α) is mainly found on mucosal surfaces, beta (β) and gamma
types are usually detected on skin surface and in hair follicles (23, 24). HPVs are classified into low and high-risk types based on their oncogenic potential (25). Low risk HPVs as a main pathogen in benign genital warts are not usually associated with any other neoplasms. Low risk HPVs include: 6, 11, 34, 40, 42, 43, 44, 53, 54, 66, 70, and 74. High risk HPV types named for their carcinogenicity, and comprised of 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 69 (26, 27). For the first time in 1992 Di Lonardo A and colleagues suggested that HPV could play a crucial role in the genesis of breast carcinoma by detecting HPV16 DNA sequence in 29.4% of breast and lymph node samples (28). Following the first publication, several more studies have documented the presence of HPV in malignant breast tumors (29-31). On the contrary negative relationship between HPV and breast cancer has been declared by few studies (32-34). Therefore, the association between HPV and breast carcinogenesis is not completely clear. Due to the high rate of breast cancer in Iranian women and probable role of high risk HPV in its carcinogenesis, we determined to investigate the presence of two high risk genotypes; HPV16 and HPV18 in our study group.

In addition, we decided to investigate presence of herpesviruses (HSV1, HSV2, VZV and CMV) in our collected BC cases, as some studies have reported a relation between a group of herpesviruses and breast cancer(35-39).

**Methods**

**Sample collection**

This cross sectional, case control study was conducted in Modarres hospital, Tehran, Iran, on cases archived in between 2008 and 2019 years. A total of 70 blocks of Formalin Fixed Paraffin Embedded (FFPE) including 59 samples diagnosed as breast carcinomas, and 11 benign breast lesions as control were collected from the pathology department archives of Modarres hospital. Also, several parameters; such as type of BC, grading of breast cancer, age and level of education were taken in written format as exclusion criteria and summarized in Table 1. All study procedures are performed in laboratories of Shahid Beheshti Medical School of sciences.

**DNA Extraction**

The genomic DNA was extracted from FFPE breast tissues. Then we performed standard polymerase chain reaction (PCR) to detect HPV DNA. In order to extract DNA, FFPE tissues were cut in 10 μm thickness by microtome. Deparaffinization was performed by adding 1 ml xylene and spinning. Afterwards we centrifuged the samples for 5 min and supernatants were removed. This step was repeated once, then 1 ml of 96% ethanol was added. Microtubes were put in 50°C heating block until ethanol was entirely dried up. Then digestion was performed by adding digestion buffer and proteinase K solution. Later, the microtubes incubated overnight, and the next day they were placed in 95 heater. Respectively phenol, phenol-chloroform, and chloroform solutions were added to samples, each step
followed by centrifugation, and subsequent removal of supernatant. Ultimately, ethanol was added, then they were kept overnight in incubator.

Samples were centrifuged at 12000 g for 30 min on the last day of extraction. After removing supernatant, the uncapped microtubes left in 37 heater to let the residual ethanol to evaporate. Finally distilled water was added and the DNA extracts were quantified with a NanoDrop spectrophotometer.

The quality of DNA extracted from paraffin-embedded tissue samples was assessed by a forward (ATGTTGCTCATGGGTGTGAA) and reverse (GGTGCTAAGCAGTTGGTGGT) primer pair targeting a sequence within the GAPDH gene. PCR amplification protocol consisted of 30 cycles of denaturation at 95 °C for 30 s; hybridization at 55°C for 30 s, and elongation at 72°C for 30 s. A final elongation step was performed at 72 °C for 10 min.

**PCR for HPV and herpes viruses**

All samples were screened for HPV L1 conserved region. The nested PCR assay was performed using two sets of primers (MY09/11 and GP5+/6+) for two consecutive amplification reactions. The first reaction was performed in 25 μl using 12.5 μl of master mix (which includes: 1X PCR buffer, 2 mM MgCl₂, 50 μM of each deoxynucleotide triphosphate (dNTP) and 2 U of Taq DNA polymerase (Takapouzist, Iran), 100-200 ng of template DNA, 10 pmol of each consensus outer degenerate 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') and distilled water. Thermal cycling (Bio Intellectica) performed with the following program: 5 min at 94 °C, 40 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, with a final extension step at 72 °C for 7 min.

The second reaction was also performed in 50 μl including 25 μl of master mix (which contains: 1X PCR buffer, 3 mM MgCl₂, 50 μM each dNTP, 2U Taq DNA polymerase), 100-200 ng of amplified DNA, and 10 pmol each inner consensus primer GP5+ (5'-TTTTGTTACTGTGGTAGATACAC-3') and GP6+ (5'-AAAAATAAACGTAAATCATATCC-3'), and distilled water. Thermal cycling used the following program: 4 min at 94 °C, 40 cycles of 1 min at 94 °C, 2 min at 40 °C and 2 min at 72 °C, with a final extension step at 72 °C for 4 min.

Negative controls containing water instead of DNA were used. We used HPV18 HeLa cell line as positive control.

Real-time PCR amplification was performed in a 20 μl volume containing 4 μl 5x HOT FIREPol® Probe qPCR Mix Plus (no ROX) (Solis BioDyne, Estonia), 0, 5 μl of each forward and reverse primers (Pishgam company, Tehran, Iran), 0.5 μl of probes (Sinaclon Co., Tehran, Iran) which listed in Table 2, and 2 μl of each sample or control, and rest of the total volume was obtained by adding distilled water. Amplification
and detection was performed by real-time PCR machine (Rotor-Gene-Q 6000 thermocycler (Corbett, Australia)). Thermal cycles used for quantification of HPV18 E6 gene and HPV type 16 E7 gene were 95°C for 12 minutes, 95°C for 15 seconds, and 60°C for 60 seconds for 40 cycles.

Duplicate reactions for each gene were performed. Probes were labeled with 6-FAM at the 5’ end and TAMRA at the 3’ end.

Each PCR was performed with negative (DNA free water) and positive controls (genomic DNA of SiHa cells for HPV-16, and genomic DNA of HeLa cells for HPV-18).

The samples were also subjected to a multiplex PCR to detect HSV-1, HSV-2, VZV and CMV. The PCR reactions were performed in a total volume of 25 μL containing 12.5 μl PCR Master Mix (10X PCR Taq polymerase buffer, 10mM of dNTPs and 1.5 U/rxn of Taq DNA polymerase [Takapouzist, Iran]), 10 pmol of each primer (HSV-1, HSV-2, CMV and VZV) (Table2), and 50 ng of genomic DNA.

mPCR (multiplex PCR) conditions were as follows: Initial denaturation (95°C for 5min), 35 cycles of denaturation (95°C for 30sec) annealing (60°C for 30 seconds) and extension (72°C for 30 seconds); Final extension was given at 72°C for 10 minutes. Samples were preserved at 4°C and then the PCR product was detected by gel electrophoresis.

**Sequencing and Phylogenetic Analysis**

The positive PCR samples were sequenced for HPV genotypes. The DNA sequence was determined with the Big-Dye terminator cycle sequencing kit and an ABI 377A sequencer (Applied Biosystems Inc.).

The HPV sequences were edited with the BioEdit program version 7.2.3, and then phylogenetic and molecular analyses were organized using MEGA software program version 6.0.6 [27]. The neighbor joining and the Kimura 2-parameters methods were used for phylogenetic reconstructions that were implemented in the MEGA 6.0.6 program. Statistical significance for the phylogenetic tree was assessed by bootstrap method (1000 replicates).

Data was analyzed by SPSS statistical software program version 16.0. The correlations were subjected to χ2 (Pearson chi-square) and Fisher's exact test. Odds ratios and logistic regression were also calculated. Statistical significance was set as a P-value less than 0.05.

**Deposit the nucleotide sequence**
The nucleotide sequences of HPV isolates that found out in this study have been settled in GenBank data base [accession numbers QED55703– QED55709]. The GenBank accession numbers for HPV6 types are: QED55703 and QED55704, and for HPV18 types are: QED55705, QED55706, QED55707, QED55708 and QED55709.

Results

Study population

HPV gene sequences were identified. Patient age range was between 29 to 81 years (mean of 50 years). Only 4 patients had low grade malignancy (6.7%), and the remaining were diagnosed with high grade (93.3%). Regarding histologic types, 5% of tumors were lobular types and remaining (95 %) were ductal carcinomas (Table 3). We reviewed the level of education of patients and we found 24% with high education level (university degree), 56% with low education level (high school diploma or less), and 20% with no education.

Assessing DNA quality

After extracting DNA from 59 FFPE breast cancer samples, and 11 benign breast lesions as control, we used PCR for GAPDH gene to check the quality of DNA samples. The amplification of GAPDH gene (fragment size of a 110 bp) was positive in all extracted DNA, indicating an adequate quality of DNA. (Fig1)

Detection of HPV and herpes viruses by PCR analysis

7 of the 59 BC samples (11.8%) were positive for HPV DNA. The outer consensus primers MY09/MY11, and GP5+/GP6+ inner primers were used in nested PCR. The length of products from first and second steps of nested PCR were 450 bp and 150 bp respectively. None of the control samples were positive for HPV DNA (Fig2).

In our study the age range of HPV positive patients were between 33 to 73 years (average age 51). All HPV positive patients had high tumor grades (II/ III). Histologic diagnosis of all positive cases was ductal carcinoma. Most of patients had low levels of education (57.1%), 14.2% had no education and the remaining had university degree (28.5%). (Table 3)

Analysis of real-time PCR supported the results of nested PCR and confirmed the presence of HPV18 in 5 breast tissue samples. HPV16 not detected in any of the cases.

Herpes simplex 1 virus sequence was detected in 8.47% (5/59) of cases using mPCR (Fig3). None of the samples were positive for HSV-2, VZV and CMV. The average age of HSV-1 positive patients was about
47 years. Most of the positive samples (80%) had high tumor grades (II/ III) (Table 3). Of total of five cases, three had low level of education, one had university degree and one had no education. We verified co-infection of HPV18 and HSV-1 in two of our cases (3.3%).

**Sequencing and construction of phylogenetic tree**

All positive PCR products were sequenced. Five out of 7 HPV positive samples were HPV18 genotype (~72%), and the rest two (~28%) were HPV6 genotype. Phylogenetic tree was shown in Fig4.

Based on our findings, the most prevalent type was HPV18, followed by HPV 6. Contrary to our expectations, we didn’t find any HPV16 genotype.

All of the HPV positive patients had invasive ductal carcinomas and high grade tumors. The age range of 30 to 40 years had three positive samples; 2 of them were HPV18 and one of them was HPV6. The age range of 50 to 60 years had one HPV6 and one HPV18 positive samples. The age range of 60 to 70 years and 70 to 80 years, each with one HPV18 positive samples.

**Discussion**

Worldwide increase in incidence and mortality of breast cancer is considerable (40). This indicates the value of identifying responsible and preventable risk factors for breast cancer. Viral agents play a crucial role as carcinogens and they are associated with nearly 20% of cancers. Development of molecular techniques and subsequent investigations has proven carcinogenic effect of High risk HPV in various tumors, including breast carcinoma (41). Based on published theories HR-HPVs trigger cancer by modifying cellular pathways; by over-expressing E6 and E7 oncoproteins and suppressing the immune system (42, 43). There are numerous publications studied the association of HPV and cervical cancer (44-46). But the correlation between HPV and breast cancer remains controversial. Lonardo A and his colleagues questioned role of HPV16 in breast cancer for the first time in 1992 by identifying DNA of this virus in 29.4% of BC samples (28). Later, various studies were conducted worldwide. In 2011, according to a meta-analysis study, the prevalence of HPV in breast cancer in Europe was the lowest (12.91%), while was the highest in Oceania (42.11%) followed by Asia (32.42%) (47). Based on their statistics and our findings, Iranian women with breast cancer are more vulnerable to HPV infection than European women but less vulnerable compared to Australian and North American women. One-fourth of Iranian women with BC are infected with HPV coincidentally. The prevalence of HPV infection in Iranian women with BC shows variation among cities/districts as follow: Shiraz (South city) 5.5%, Mazandaran (Northern district) 25.9%, Kermanshah (western district) 62.1%, Mashhad (Northeast city) 26.2%, and Tehran (Capital) 5.7%. (48-52). In this study, we surveyed women with BC in Tehran and our result showed the 11.8% prevalence of HPV. Our results are similar to the findings of a study conducted in the United States in 2019, which
used multiplex PCR with HPV E6 or E7 gene-specific primers for 64 serum samples of BC patients. So they reported 5 BC patients with positive HPV DNA (7.8%) (53). Similarly, a study performed in 2011, detected 4 HPV DNA from 62 tumor tissue samples (6.5%) (54). In contrast, different reports from the United States, the United Kingdom and Venezuela showed a higher percent of HPV prevalence in BCs; such as 86%, 42% and 41.6%, respectively (29, 55, 56). On the other hand, several studies performed in Denmark, Australia, India, etc. couldn’t find any association between the presence of HPV and breast cancer (32, 33, 57). There are studies declared that HPV6 (low risk HPV) was the most common type of virus among their specimens (30, 58) since we also found HPV6 in our samples so we recommend to consider low risk HPVs in breast cancers studies. Our result was similar to N Khodabandehlou et al., (59) and JS Lawson et al., (60), which reported HPV18 as the most prevalent type in positive samples. Noticeably, we did not find HPV type 16, which was demonstrated to be prevalent in previous studies (61, 62).

Differences in results may be due to variation in patient age range, geographical distribution of viruses, techniques and the quality of samples used in each study. According to a few published papers, the mean age of breast cancer in Iranian women is between 47.95 to 54.6 years. (63-66). In our study, the age range of patients was from 29 to 81 years (mean of 50 years) while according to a study in 2008 in Syrian women, the age range was 26 to 66 (with median age of 52) with higher prevalence of HPV (61.06%) (67). Also, in our study, the age range of HPV positive patients were from 33 to 73 years with a viral prevalence of 11.8%, but in a study in Venezuela the age range was 51 to 60 years with a viral prevalence of 41.67% (55). Therefore, the age range can be considered as an important issue in the rate of viral detection.

BC is often labeled as a “welfare disease” Because the incidence rate was reported higher in women in Western societies with higher social class and higher education. Of course, this can be related to timely diagnosis and regular checkups, as well as better training in this group (68).

But in 2017, J Bahk and colleagues conducted age-period-cohort (APC) analysis in Korea. They reported the BC mortality was higher in women with a higher education than in women with no education or a primary education during 1983-1992, and the reverse was true in 1993-2012 (69).

In this study, low level of education found in 56%, and no education in 20% of patients. And in HPV positive patients, 57.1% had low levels of education and 14.2% had no education. So due to our results, most of BC patients and HPV positive patients had low levels of education. Therefore, it can be noted that education, training and socio-economic level of individuals can play a significant role in the prevention and timely treatment of breast cancer.

In this study, regarding histologic types, 95% of the total tumor specimens were ductal carcinoma and 5% were lobular carcinoma. And all positive HPV samples were ductal carcinoma. Similarly, in 2019, N Khodabandehlou et al. showed ductal carcinoma as the most frequent type in their HPV positive patients (59).
In addition, in UK base study, NA Salman reported higher prevalence of HPVs in patients with invasive ductal carcinoma\(^{56}\). From these studies, it can be concluded that mammary ducts provide an entry point for HPV infection\(^{70}\).

Few studies surveyed the relation of tumor grades and HPV positive breast cancers. As the study of C Kroupis and et al. which recorded high grade tumor in 70.6% of HPV-positive breast cancer cases \(^{61}\). In our study, all of positive samples had a high grade tumor.

Ni Li and colleagues stated a remarkable point about the type of samples collected for studies. They showed higher prevalence of HPV DNA in FFPE samples than fresh samples \(^{47}\) According to their findings the age of specimen also may affect on prevalence of virus detection. Our results indicated that samples collected earlier (from 2018 to 2019) showed higher HPV positivity compared to older collected samples (from 2008 to 2018).

Besides, due to the important role of herpesviruses as a co-factor in the oncogenesis of BC \(^{39, 71, 72}\), we decided to detect this virus in our specimens. In 2013, BM Khashman et al. detected HSV-1 Ag with 31.8% prevalence using direct immunofluorescence in FFPE breast tissues \(^{36}\). We suggest utilizing a different technique, since it may effect results. In the literature there are several studies investigating the relationship between HPV and herpesviruses \(^{73, 74}\). So we used multiplex PCR with different primers for HSV-1, HSV-2, CMV and VZV. We detected only HSV-1 with 8.47% prevalence. Using individual immunofluorescence method for each virus might be more specific and provide higher accuracy.

These data determine further evidence of a possible role for Human papillomavirus and Herpesvirus in breast carcinogenesis, however additional studies in larger patient population and by techniques with higher specificities can ascertain our results. Also for early detection of cervical cancer, the American cancer society guidelines recommend Pap test and HPV DNA testing. Currently, the American cancer society recommends mammography for regular breast cancer screening, while further studies on the association between breast cancer and HPV in the future may add HPV screening to this guideline \(^{75}\).

**Conclusion**

Overall, in the present study, we identified HPV18 and HPV6 genotypes in 11.8% of FFPE samples from women with breast cancer, and HPV18 was the most common genotype. Most of positive samples were at grade II and III. Also HSV-1 was detected in 8.47% of BC samples. And coinfection of HPV and HSV-1 was declared in 3.3% of them. More epidemiological and molecular studies with large samples are needed to validate the association of breast cancer and HPV genotypes, and role of coinfection of HPV and herpesviruses in carcinogenesis of breast cancer.

**Declarations**

*Ethics approval and consent to participate*
The study was approved by ethical committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran under the Ethics code of IR.SBMU.MSP.REC.1397.49

**Consent for publication**

Not applicable.

**Availability of data and material**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

Morvarid Golrokh Mofrad collected the samples and analyze them, and wrote the manuscript; Zohreh Azita Sadigh, Sanaz Ainechi and Ebrahim Faghihloo revised the manuscript. All authors read and approved the final manuscript.

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**Abbreviations**
Human papillomavirus (HPV), breast cancer (BC), herpes simplex virus (HSV), cytomegalovirus (CMV), varicella zoster virus (VZV), high risk (HR), low risk (LR), Formalin Fixed Paraffin Embedded (FFPE), polymerase chain reaction (PCR), multiplex polymerase chain reaction (mPCR), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), base pair (bp), minute (min), distilled water (dH2o).

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Tables

Table 1. Clinical and Pathological Features of all cases

|                        | Tumor cases | control cases |
|------------------------|-------------|---------------|
| Numbers                | 59          | 11            |
| Age ranges             | 29 to 81 years | 33 to 69 years |
| Histological types     | 5% lobular  | benign breast lesions |
|                        | 95% ductal  |               |
| Grade                  | 6/8% Low grade | 93/2% High grade |
| Levels of education    | 56% low level of education | 54% low level of education |
|                        | 24% high level of education | 46% high level of education |
|                        | 20% no education |               |

L: Low grade (I), H: High grade (II/ III)

Table 2. List of Primers used for GAPDH PCR, nested PCR, real-time PCR and multiplex PCR

| Assay       | Name  | Sequence (5’ → 3’) |
|-------------|-------|--------------------|
| GAPDH       | GAPDH-F | ATGTTCGTCATGGGTGTGAA |
|             | GAPDH-R | GGTGCTAAGCAGTTGGTG |
| MY09/11     | MY09   | CGTCC (A/C) A (A/G) (A/G) GGA (A/T) |
| ACTGATC     | MY11   | GC (A/C) CAGGG (A/T) CTATAA(C/T) AATGG |
Table 3. Clinical and Pathological Features of positive cases

| Tumor cases | control cases | tumor grade | histological type |
|-------------|---------------|-------------|------------------|
| **High (II/ III)** | **Lobular/ductal** | **6/8% L, 93/2% H** | **5% lobular, and 95% ductal** |

**Total cases**

- **ductal**
  - **Total cases**: 59, 11, 6/8% L, 93/2% H, 5% lobular, and 95% ductal

- **HPV-positive**: 7, 0, 100% H, 100% ductal
- **HPV-negative**: 52, 11, 7/7% L and 92/3% H
|                      | Count | Count   |                  |                      |
|----------------------|-------|---------|-----------------|----------------------|
| HSV-1-positive       | 5     | 0       | 80% H and %20 L | 100% ductal          |
| HSV-1-negative       | 54    | 11      | 5/6% L and 94/4% H |
| HSV-2-positive       | 0     | 0       |                 |                      |
| HSV-2-negative       | 59    | 11      |                 |                      |
| VZV-positive         | 0     | 0       |                 |                      |
| VZV-negative         | 59    | 11      |                 |                      |
| CMV-positive         | 0     | 0       |                 |                      |
| CMV-negative         | 59    | 11      |                 |                      |

L: Low grade (I), H: High grade (II/ III)