Research Square

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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

Keywords: human papillomavirus, hpv, breast cancer, genotype, herpes virus, pcr

DOI: https://doi.org/10.21203/rs.3.rs-38630/v2

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Abstract

Background: The role of viruses in the aetiology of breast cancer (BC) have been significantly investigated in recent years. Human papillomavirus (HPV) has been detected in invasive breast carcinomas, and most studies have only focused on the detection of viral DNA therefore the aim of this study was to determine the genotypes of each papillomaviruses.

Methods: We collected and analyzed 70 blocks of Formalin Fixed Paraffin Embedded (FFPE) including 59 BC samples, and 11 benign breast lesions as control from Iranian patients using nested PCR. Real-time PCR supported 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, but we didn't find HPV16 in these cases. All HPV positive patients had high tumor grades (II/ III), and the age range of them were from 33 to 73 years (average was about 51). Most of HPV positive patients had low levels of education. Histologic diagnosis of all positive cases were ductal carcinoma. On the other hand 5 of 59 BC specimens (8.47%), were positive for HSV-1.

Conclusions: Our results suggest that high risk HPVs may play a role in breast carcinogenesis. Also we detected HPV6 and HSV1 in our BC samples, taking into account a carcinogenesis role for low risk HPVs, in addition to high risk HPVs. 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 it's 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 the incidence rate of BC is higher in developed countries, ratio of mortality in developing countries is higher. More than 55% of breast cancer related deaths occur in the developing countries (3, 4). 5 year survival has risen in most developed countries, but the low survival rates in developing countries are related to lack of immediate detection programs, adequate diagnosis and treatment facilities (5, 6). In Iran breast cancer is the fifth most common cause of death related to cancers (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). On the other hand, the pathogenesis role of some infectious agents including viruses has been evaluated. (11-14).

Many studies have shown the role of human papillomavirus (HPV) in different cancers (15-17). And over the past two decades various studies have suggested the role of HPV in breast cancer (18-22).

Over 200 HPV types have been identified and most of them fall within three genera: alpha (α), beta (ß) and gamma (γ). Alpha (α) mainly found on mucosal surfaces, beta (ß) and gamma (γ) are generally found on skin and hair follicles (23, 24). HPVs are classified into low and high-risk types based on their oncogenic potential (25). Low risk HPVs although not usually associated with cancer development, can cause benign genital warts. The HPV types that have the ability to cause cancer are considered high risk. Low risk HPVs are including: 6, 11, 34, 40, 42, 43, 44, 53, 54, 66, 70, and 74. High risk HPV types are 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 study, several studies have shown the presence of HPV in malignant breast tumors (29-31). Conversely, conflicting studies were declared the negative relationship between HPV and breast cancer (32-34). So the association between HPV and breast carcinogenesis is not yet clear completely. Due to the high rate of breast cancer in Iranian women and the role of high-risk genotypes of papillomaviruses in carcinogenesis, this study was conducted to determine the presence of two high-risk genotypes HPV16 and HPV18 in malignant breast tissue.

The association between some herpesviruses and breast cancer have been studied before (35-39). In this regards, we also investigated the presence of herpes simplex 1 and 2 (HSV-1 and HSV-2), varicella zoster virus (VZV or HSV-3) and cytomegalovirus (CMV or HSV-5) in the collected BCs samples.

Methods

Tissue samples

This cross sectional, case control study was performed between 2008 and 2019 in the Modarres Hospital (Tehran, Iran). 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. The specimens were carried to the school of Medicine in Shahid Beheshti University for the following studies.

DNA Extraction for PCR

The genomic DNA was extracted from FFPE breast tissues. Then we performed standard polymerase chain reaction (PCR) to detect HPV DNA. For extraction at first, 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 50ºC heating block until ethanol was entirely dried up. Then the digestion was performed by digestion buffer and proteinase K solution. At the end they incubated overnight, and next day they were placed in 95ºC heater. Afterwards, phenol, phenol-chloroform

and only chloroform solution were added respectively followed by centrifugation and subsequent removal of the supernatant. Ultimately, ethanol was added, then they were kept in the incubator overnight.

In the last day of extraction samples were centrifuged at 12000 g for 30 min. Then threw away supernatant and left the ethanol to evaporate in 37ºC heater. Finally distilled water was added and then the DNA extracts were quantified with a NanoDrop spectrophotometer.

GAPDH PCR amplification

The quality of DNA extracted from paraffin-embedded tissue samples was assessed by using a forward (ATGTTCGTCATGGGTGTGAA) 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 30s. A final elongation step was performed at 72 °C for 10 min.

HPVdetection by nested PCR

All samples were screened for the 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 $MgCl_2$, 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 $MgCl_2$, 50 µM each dNTP, 2U Taq DNA polymerase), 100-200 ng of amplified DNA, and 10 pmol each inner consensus primer GP5**+ (5’-TTTGTTACTGTGGTAGATACTAC-3’)** and GP6+ **(5’-AAAAATAAACTGTAAATCATATTC-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.

Sequencing and Phylogenetic Analysis

The positive PCR samples were sequenced for HPV genotyping. 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).

Nucleotide Sequence Accession Number

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.

Real-time PCR

The 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 Table2, 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).

Multiplex PCRfor 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.

Statistical analysis

Data was analyzed by SPSS statistical software program version 16.0. The correlations were subjected to $\chi 2$ (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.

Results

Study population and include criteria

HPV gene sequences were identified. The age ranges of patients were from 29 to 81 years (mean of 50 years). In our study we had 4 patients (6.7%) with a low grade malignancy (tumor grade I), and all remaining had high grade tumor (II and III) (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. High level of education (having university's degree) found in 24% of patients, low level (having diploma or less) in 56%, and no education in 20%.

Assessing DNA quality from FFPE samples

After extraction of genomic DNA from 59 FFPE breast cancer samples, and 11 benign breast lesions as control, we used PCR for GAPDH gene to check the accuracy of DNAs. 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 DNA in samples using nested PCR

7 of the 59 BC samples (11.8%) were positive for HPV DNA. The length of product from first and second step of nested PCR using the outer consensus primers MY09/MY11, and the inner primers GP5+/GP6+ 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 from 33 to 73 years (average is about 51). All HPV positive patients had high tumor grades (II/ III). Most of patients had low levels of education (57.1%), 14.2% had no education and remaining had universities degrees (28.5%). Histologic diagnosis of all positive cases was ductal carcinoma. (Table 3)

Detection of HPV18 DNA in samples using real-time PCR

Analysis of real-time PCR supported the results of nested PCR and confirmed the presence of HPV18 in 5 breast tissues. And no positivity for HPV16 were detected.

Sequencing HPV positive samples and constructing 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 Fig 3.

According to our results, the most prevalent type was HPV18, followed by HPV 6. Contrary to our expectations, we didn't find HPV16 in cases.

All of HPV positive patients with different genotypes, had invasive ductal carcinomas and high tumor grades. 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.

Detection of HSV-1 using Multiplex PCR

Herpes simplex 1 virus sequences were detected in 8.47% (5/59) of cases (Fig4). 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). Three of the patients had high school diploma, one bachelor degree and the last one had no education. Therefore, we verified that two women (3. 3%) had HPV18 and HSV-1 coinfection.

Discussion

Incidence and mortality rates of breast cancer are enhancing considerably worldwide (40). This indicates the value of identifying risk factors that are responsible and preventable for breast cancer. Viral agents play a crucial role as a risk factor in cancer development as they are associated with nearly 20% of cancers. High risk HPVs, which can cause different cancers, based on development of molecular techniques and subsequent investigations, are also detected in breast cancer (41). Various theories indicate HR-HPVs can induce cancer by modifying cellular pathways and inducing tumorigenesis by E6 and E7 genes and suppressing the immune system (42, 43). There are numerous publications studied the association of HPV and cervical cancer (44-46). But the correlation of HPV and breast cancer remains controversial. Lonardo A and his colleagues considered the correlation of HPV16 in breast cancer for the first time in 1992, after they found this virus DNA in 29.4% of BC samples(28). Later, various studies were conducted around the world. In 2011, according to a meta-analysis study, the prevalence of HPV in breast cancer in Europe was the lowest (12.91%) but 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.

Conclusion

Overall, in the current study we showed the presence of HPV18 and HPV6 genomes in FFPE tissue Samples in women with breast cancers in Tehran (11. 8%). 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.

Funding

This article is financially supported by “Research Department of the School of Medicine Shahid Beheshti University of Medical Sciences” (Grant No 14847).

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.

Acknowledgements

Not applicable.

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 95% ductal	benign breast lesions
Grade	6/8% Low grade 93/2% High grade	

Levels of education	56% low level of education 24% high level of education 20% no education	54% low level of education 46% high level of 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
(110 bp)	GAPDH-R	GGTGCTAAGCAGTTGGTGGT
MY09/11	MY09	CGTCC (A/C) A (A/G) (A/G) GGA (A/T) ACTGATC
(450 bp)	MY11	GC (A/C) CAGGG (A/T) CTATAA(C/T) AATGG
GP5+/GP6+	GP5+	TTTGTTACTGTGGTAGATACTAC
(150 bp)	GP6+	AAAAATAAACTGTAAATCATATTC
HPV 18 E6	HPV 18 E6-F	CTG GGC ACT ATA GAG GCC AGT
(78 bp)	HPV 18 E6-R	GTG TTT CTC TGC GTC GTT GG
	HPV 18 E6-P	[FAM] -TGCAACCGAGCACGACAGGAACGA - [TAMRA]
HPV 16 E7	HPV 16 E7-F	GAG GAG GAG GAT GAA ATA GAT GGT
(98 bp)	HPV 16 E7-R	AGC GTA GAG TCA CAC TTG CAA CA
	HPV 16 E7-P	[FAM] -CTCTGTCCGGTTCTGCTTGTCCAGCT- [TAMRA]
HSV-1	HSV-1-F	GACTCTCCCACCGCCATCAG
(269 bp)	HSV-1-R	TGTCTTCGGGCGACTGGT
HSV-2	HSV-2-F	TATGCCTATCCCCGGTTGGA
(715 bp)	HSV-2-R	CGTGCCATCCGAATAAACGTG
VZV	VZV-F	TTGTGTCGGTCTCTCCAAGC
(934 bp)	VZV-R	TACGTCTTCAACCTCACGCC
CMV	CMV-F	TGGCTTTTCTTGAACGTGCG

(716 bp)	CMV-R	CCTTGACGCTGGTTTGGTTG

F: Forward primer, R: Reverse primer, P: Probe, FAM: 6-carboxy fluorescein, TAMRA: 6-carboxy
tetramethyl rhodamine, bp: base pair

Table 3. Clinical and Pathological Features of positive cases

	Tumor cases	control cases	tumor grade Low (I)/ High (II/ III)	histological type lobular/ 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	
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)

Figures

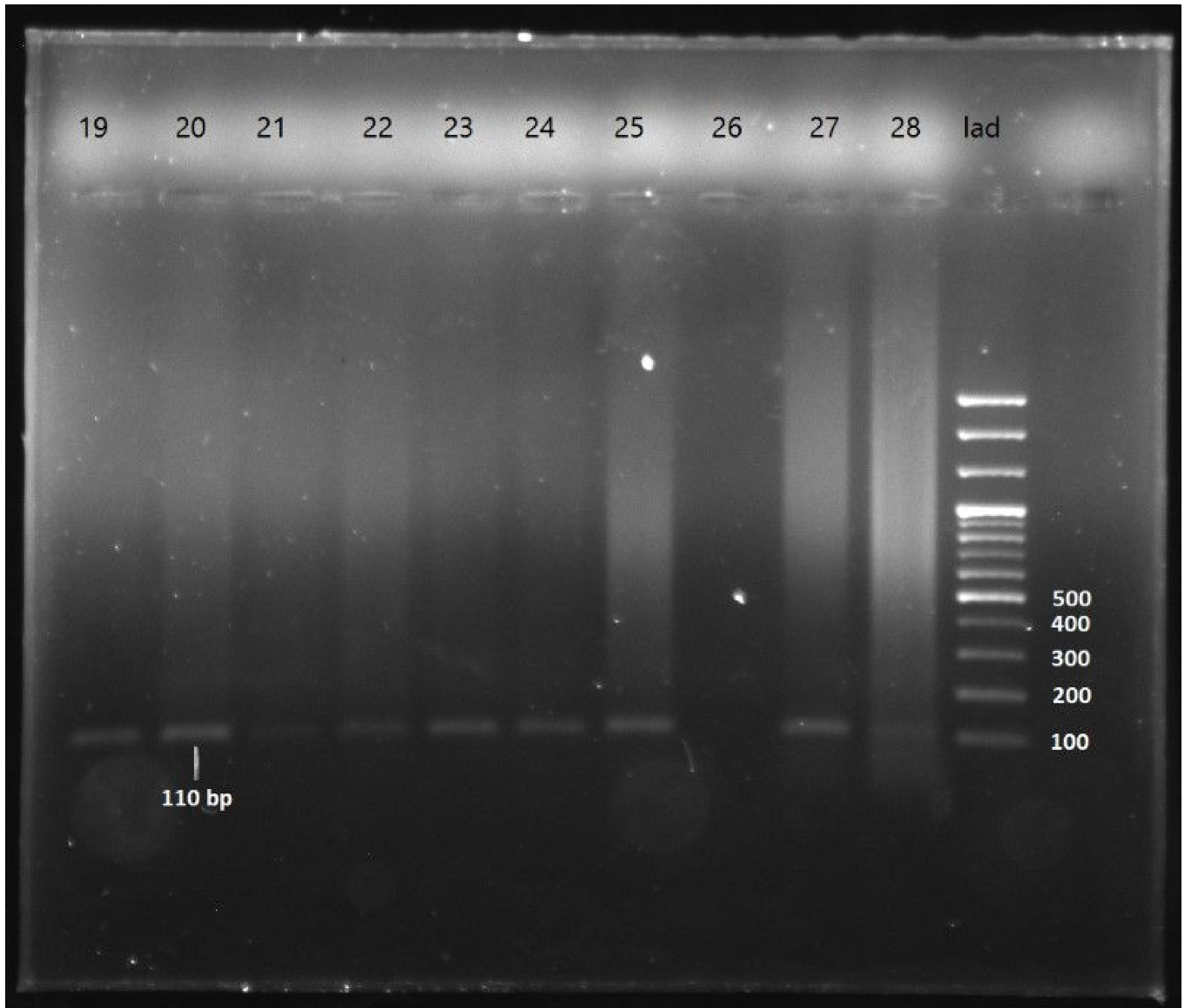

Figure 1

An agarose gel electrophoretogram of amplified GAPDH after the PCR as described in the materials and methods. Lanes 19 to 28 breast cancer samples, last lane: 100-bp DNA ladder.

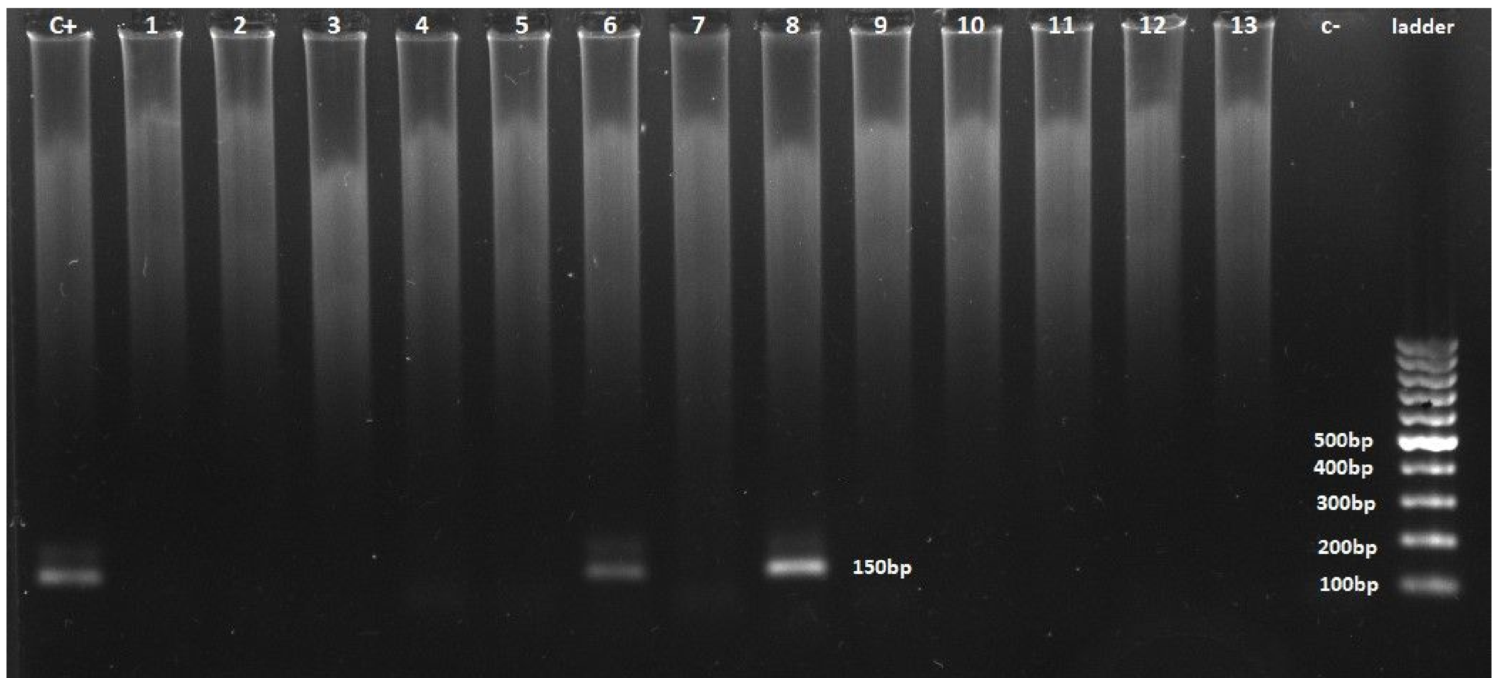

Figure 2

An agarose gel electrophoretogram of amplified DNA after the nested PCR as described in the materials and methods. C+ Lane: positive control (hpv18 HeLa cell line), Lanes 1 to 13 breast cancer samples, c- lane: negative control, last lane: 100-bp DNA ladder.

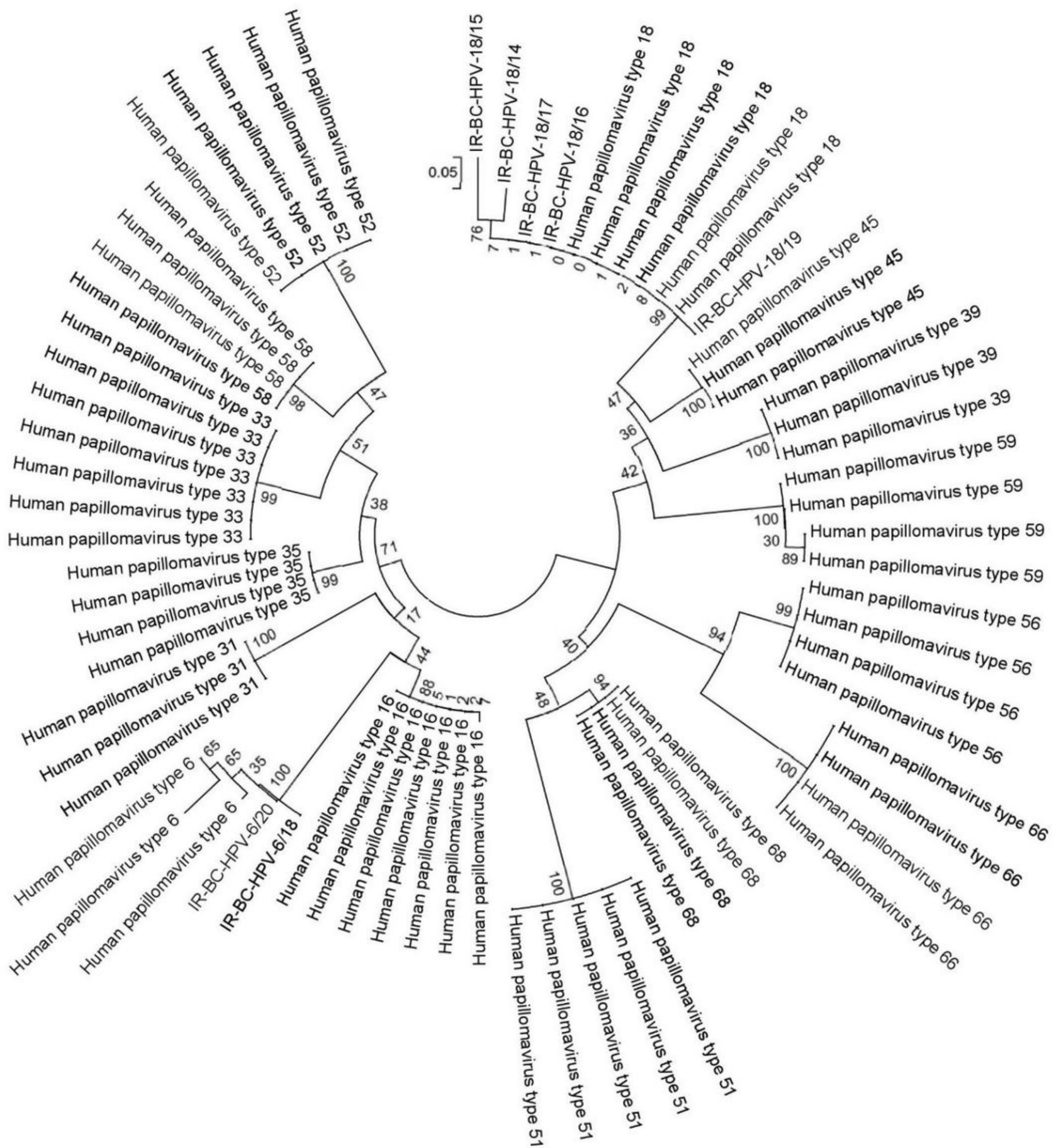

Figure 3

Phylogenetic trees of HPV16 and HPV18 Based on L1 Nucleotide was Constructed Using neighbor joining method and the Kimura 2-Parameter model by MEGA 6 package.

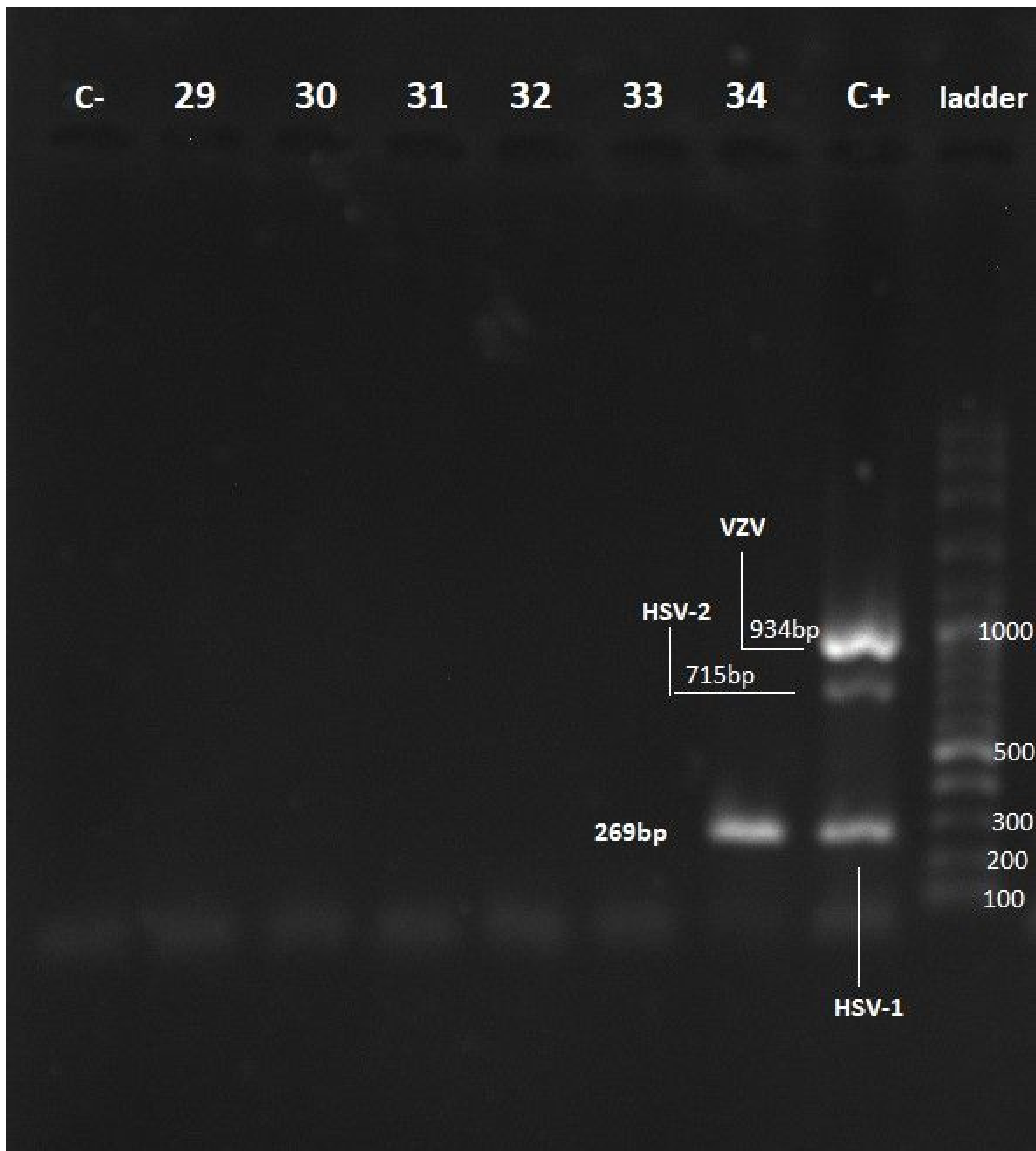

Figure 4

Gel electrophoresis of the multiplex PCR amplification products. Lanes 29 to 34 breast cancer samples. The amplicon length was 269bp.c+ lane: positive control, c- lane: negative control, last lane: 100-bp DNA ladder.

Supplementary Files

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- nanodroppicture.jpg