Iranian Journal of Microbiology
Volume 15 Number 4 (August 2023) 585-593

ORIGINAL ARTICLE

Low presence of papillomavirus and its lack of correlation with clinicopathological factors in breast cancer: a case control study

Manoochehr Makvandi[1,2], Mohammad Rashno[3], Ebrahim Faghihloo[4], Shahram Bagheri[5], Saeed Hesam[6], Habibollah Mirzaei[1,2], Ali Ramezani[1,2*]

[1]Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2]Department of Virology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[3]Cellular and Molecular Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[4]Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[5]Department of Pathology, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[6]Department of Biostatistics and Epidemiology, Faculty of Public Health, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Received: February 2023**, Accepted:** July 2023

ABSTRACT

Background and Objectives: Breast cancer is currently the most commonly diagnosed neoplasm in women worldwide. There is evidence that human papillomavirus (HPV) infection may play a key role in breast cancer aggressiveness, but results are conflicting across studies. The aim of this study was to investigate the presence of the HPV viral genome in benign and malignant breast tissue samples and its clinicopathological characteristics of cancer.

Materials and Methods: In this case-control study, 100 formalin-fixed paraffin-embedded (FFPE) of breast cancer and 100 blocks of non-cancerous breast tissue were selected as a control group from the pathology department of Imam Khomeini Hospital in Ahvaz from 2020-2022. The presence of HPV was detected using nested PCR including MY09/11 primers and sequencing were performed for virus genotyping.

Results: The present study enrolled 100 subjects each in two cancer and control groups with a mean age of 52.81±13.23 and 35.77±11.65, respectively. The risk of cancer in HPV-infected patients is almost 5 times higher than in HPV-negative individuals, it is not statistically significant (OR =4.99, 95% CI 0.35 to 72.15, p=0.238). The prevalence of HPV in the cancer and control groups was 7% and 1%, respectively and HPVs detected in two groups were of the HPV 16 genotype. Although the chance of ER and PR expression, lymphvascular involvement, perineural invasion, and higher tumor grade was higher in HPV-positive subjects than in HPV-negative subjects, this was not statistically significant (OR>1, p>0.05).

Conclusion: Based on studies reporting the existence of sequences of different high-risk HPV types (oncogenes) in breast cancer tissues, this study confirmed the hypothesis of a possible infectious cause in the development of breast cancer. So far, however, the results have been controversial and inconclusive. Further studies with large sample sizes are needed to demonstrate the link between HPV and breast cancer.

Keywords: Breast malignant tumor; Human papillomavirus; Prognostic factors; Nested polymerase chain reaction; Prevalence

*Corresponding author: Ali Ramezani, PhD, Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; Department of Virology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Tel: +98-6133738313 Fax: +98-6133738313 Email: Ramezani.al@ajums.ac.ir

INTRODUCTION

Breast cancer is the leading cause of cancer in women, with an estimated 2.3 million new breast cancer cases and 685,000 breast cancer deaths worldwide in 2020. Statistical analysis shown that the incidence and mortality rate of breast cancer is increasing (1). In Iran, breast cancer is the fifth leading cause of death among Iranian women and 76% of all common cancers in women are due to breast cancer (2). Although many types of proven and controversial risk factors for breast cancer have been reported, but some risk factors such as gender, aging, estrogen, family history, gene mutation, unhealthy lifestyle, and infectious agents (viruses) could increase the likelihood of breast cancer progression to cancer (3). Infectious agents are responsible for 18% of human cancers, and among these, oncogenic viruses are involved in 20% of human cancers. So far, viruses such as Epstein-Barr virus (EBV), human herpesvirus type 8 (HHV-8), cytomegalovirus (CMV), human papilloma virus (HPV) and herpes simplex virus type 1 (HSV-1) have been detected in the breast cancer (4). Several studies have shown that EBV, HPV and MMTV may play an important role in the development of breast cancer, but their mechanisms are still very controversial and unclear (5). HPV is a small circular double-stranded DNA virus classified into two types of mucosal and skin viruses. Mucosal HPV includes two categories, low-risk types (HPV-6 and HPV-11) and high-risk types (HPV 16, 18, 31, 33, 45). High-risk HPV types are usually associated with malignant tumors of epithelial cells and have been found in 99% of cervical cancers (6). It is well known that persistent infection with high-risk HPV types can lead to cancer of the cervix, vagina, vulva, penis, anus, head and neck, and oropharynx (7). Still, reports of the relationship between HPV and breast cancer are controversial. The prevalence of HPV in breast cancer tissues in different geographic regions of the world has been reported from 0 to 86% (8). The oncogenic mechanisms of HPV causing cervical cancer have been studied extensively, and on the other hand, the biology of HPV in breast cancer is almost the same as that of HPV in cervical cancer. High-risk HPV types encode a range of proteins, either early (E1-E7) or late (L1 and L2). Although all viral proteins are involved in viral replication, few early viral proteins are involved in cell transformation. Oncoproteins E6 and E7 play key roles in cell transformation. E6 and E7 disrupt cell cycle regulation by binding or inhibiting cellular antitumor factors such as P105RB and P53, respectively, which ultimately cause cell death (9). Previous studies have shown that immortalization of normal human mammary epithelial cells in vitro by E6 and E7 genes is consistent with the role of HPV in breast cancer (10). Considering the challenging role of HPV in breast cancer and also the difference in the reported prevalence of this virus in studies, therefore, this study was conducted with the aim of examining the presence of HPV viruses in two groups of breast cancer samples (as cases) and non-cancerous samples (as controls) and also to determining the association between HPV infection and clinicopathological features of breast cancer.

MATERIALS AND METHODS

Study population and sample collection. In this case-control study, 100 paraffin blocks of breast cancer and 100 formalin-fixed paraffin-embedded (FFPE) blocks of non-cancerous breast tissue were selected as a control group from the pathology department of Imam Khomeini Hospital in Ahvaz from 2020-2022. Samples were collected after receiving the code of ethics by the Ethics Committee of Jundishapur University of Medical Sciences in Ahvaz (IR.AJUMS.REC.1398.232). Using a microtome, very thin slices of 5-10 um were prepared from paraffin blocks and placed in 1.5 ml Eppendorf microtubes without DNase/RNase. Before cutting each block, special care was taken to change gloves and scalpel to avoid contamination between different samples. FFPE block sections and hematoxylin and eosin stains were performed to confirm the diagnosis and to mark areas for microdissection by a pathologist.

DNA extraction protocol and quality. First, 5-10 µm sections were deparaffinized by incubating at 37°C for 1 hour with 1 ml xylene, followed by washing with decreasing concentrations of ethanol (100%, 70% and 50%) for 30 minutes. After the plates were dried at 37°C, 300 µl of lysis buffer, 30 µl of proteinase K were added to each sample for lysis and incubated at 37°C overnight (Bioneer, Korea). Proteins were removed from the suspension using equal volumes of phenol, chloroform, isoamyl alcohol (Sigma-Alrdich, USA) and the extracted DNA was precipitated with

absolute ethanol. The concentration and purity of the extracted DNA was evaluated by Nanodrop. To check the quality and integrity of the extracted DNA, the 110 bp human globin gene product was amplified by PCR using forward primer PCO3: 5'-ACACAACT-GTGTTCACTAGC-3' and reverse primer PCO4: 5'-CAACTTCATCCACGTTCACC-3 (Table 1) (11). Finally, only beta globin positive samples were used for HPV detection.

HPV detection. The presence of HPV was detected using a nested PCR including MY09/11 primers (outer primers) and GP5+/6+ primers (inner primers) from the L1 region. Primer set MY09/MY11 [MY9 (5' - CGT CCA/C AA/GA/G GGA A/TAC TGA TC - 3')] and [MY11 (5' - GCA/C CAG GGA/T CTA TAA C/ TAA TGG – 3), can amplify a wide range of HPV types to produce a 450 bp fragment. The second round of PCR was performed using GP5+/GP6+ primers. The GP5+/GP6+ primer set [GP5+ (5'- TTT GTTACT GTG GTAGATACTAC-3')] and [GP6+ (5'-AAA AAT AAA CTG TAA ATC ATA TTC-3')] is able to identify a wide range of HPV types using lower annealing temperature during PCR and produced a 150 bp fragment (Fig. 1) (12). The PCR protocol was performed as previously described in the study of Mirzaei et al. (13). PCR products to determine the presence of HPV in the samples were checked under 1.5% gel electrophoresis and observed using a UV transilluminator. The PCR was repeated to ensure results in positive HPV samples.

Sequencing. To confirm the results and to detect the HPV type two directions sequencing was performed on HPV positive PCR products using primers MY09/11 by an ABI Applied Biosystems 3130xl

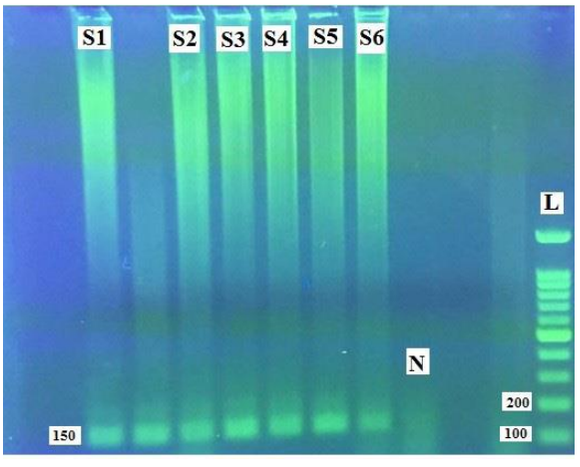

Fig. 1. Analysis PCR of HPV, positive samples Lane 1-6, Lane 7 Negative, Lane L: 100-bp size marker

Table 1. Frequency and association Demographic, clinical information with cancer

		Cancer group	Benign group	Univariant analysis			Multivariant analysis		
				OR	95% confidence interval (CI)	P-value	OR	95% confidence interval (CI)	P-value
Age		52.81±13.23	35.77±11.65	1.13	(1.09-1.17)	<0.001	1.14	(1.09-1.19)	<0.001
Marital status	Married	43 (43)	83 (83)						
	Single	47 (47)	11 (11)	8.25	(3.86-17.51)	<0.001	9.21	(3.15-26.88)	<0.001
	Divorced	10 (10)	6 (6)	3.22	(1.09-9.44)	0.033	2.75	(0.74-10.26)	0.132
Location	City	66 (66)	83 (83)						
	Rural	34 (34)	17 (17)	2.52	(1.29-4.89)	0.007	2.93	(1.16-7.43)	0.024
Menopause	Post	52 (52)	43 (43)	1.48	(0.83-2.65)	0.183	1.38	(0.59-3.25)	0.46
	Pre	42 (42)	51 (51)						
	unknown	6 (6)	6 (6)						
Smoking	Yes	8 (8)	8 (8)	1.10	(0.39-3.14)	0.854	1.59	(0.34-7.46)	0.556
	No	88 (88)	84 (84)						
	unknown	4 (4)	8 (8)						
History of cancer	Yes	44 (44)	11 (11)	6.36	(3.03-13.33)	<0.001	3.80	(1.39-10.41)	0.009
	No	56 (56)	89 (89)						
History infection	Yes	7 (7)	2 (2)	3.69	(0.75-18.21)	<0.001	5.84	(0.51-67.26)	0.157
	No	93 (93)	98 (98)						
HPV	Positive	7 (7)	1 (1)	7.45	(0.9-61.37)	0.063	4.99	(0.35-72.15)	0.238
	Negative	93 (93	99 (99)						

sequencer (Applied Biosystems, Foster City, CA) and analyzed using SnapGene (GSL Biotech) software. Sequences were registered in the GenBank database and accession numbers obtained (OL688621-OL688628).

Phylogenetic analysis. 8 positive HPV-16 L1 sequences were blasted in the web software www.ncbi.nlm.nih.gov/BLAST and a phylogenetic tree was drawn using the Neighbor joining method using Mega 5.0 software.

Statistical analysis. For qualitative variables, frequency, percentage and descriptive statistics including mean index and standard deviation were used for quantitative variables. Missing data were imputed. To treat cell number zero, the method of the article by Dureh et al. was used (14). The data were analyzed in two parts, univariate and multivariate. In the multivariate analysis, other variables were controlled to measure the relationship between an independent variable and a response variable. Logistic regression, rank and linear logistic regression were used to analyze the data. All statistical tests were performed in SPSS version 22. Differences were considered significant when the p-value was less than 0.05.

RESULTS

The present study enrolled 100 subjects each in two cancer and control groups with a mean age of 52.81±13.23 and 35.77±11.65, respectively. Table 1 shows the sociodemographic data, the distribution of histopathological types and the HPV test results in two groups. The distribution of the most commonly diagnosed histopathologic type in the malignancy group was invasive ductal carcinoma (IDC) with a frequency of 70 (70%), followed by ductal carcinoma in situ (DCIS) with a frequency of 25 (25%), while fibroadenoma and fibrocystic were the most common histopathologic types in the benign group, with frequencies of 40 (40%) and 23 (23%), respectively. HPV DNA was detected in 7 cases from the malignant group and 1 case from the benign group. All HPVs identified in two groups were of the HPV 16 genotype. Cancer risk in the presence of demographic data, socio-demographic data, and HPV results is presented in Table 2. Based on a multivariate analysis, the risk of cancer increases significantly by 1.14-fold for a one-unit increase in age (OR =1.14, 95% CI 1.09 to 1.19, p<0.001). Analysis using crude odds ratios (ORs) showed that single individual had a cancer risk about 9-fold higher than married individual, which is statistically significant (OR =9.21, 95% CI 3.15 to 26.888, p<0.001). The risk of cancer in individuals with a family history of cancer was significantly 3.80-fold higher than in individual without a history of cancer (OR =3.80, 95% CI 1.39 to 10.41, p=0.009). In relation to place of residence, the risk of cancer in individuals living in rural areas is significantly almost three-fold higher than in individuals living in urban areas (OR =2.93, 95% CI 1.16 to 7.43, p=0.024). With regard to other sociodemographic data, no significant association with cancer risk was found. Although the risk of cancer in HPV-infected patients is almost 5 times higher than in HPV-negative individuals, it is not statistically significant (OR =4.99, 95% CI 0.35 to 72.15, p=0.238). The comparison of clinicopathological characteristics using univariate and multivariate analysis between HPV-positive and HPV-negative individuals is presented in Table 2. The chance of ER expression, PR, lymphovascular involvement, perineurial invasion, and higher tumor grade is higher in HPV-positive individuals than in HPV-negative individuals (OR>1), while the chance of lymph node involvement, surgical margin involvement, Ki-67 expression, and of HER2 expression in individuals infected with HPV is less than uninfected individuals (OR<1), although no statistically significant association has been found (p>0.05). Eight of the HPV-16 cases were sequenced and clustered with the virus from different regions of the world, including as Brazil, Thailand, USA, Ireland and China (Fig. 2).

DISCUSSION

Breast and cervical cancer are the leading cause of death in women, particularly in developing countries around the world. HPV is the cause of more than 5% of all human cancers (15). 99.7% of cervical cancer cases, 50% of head and neck squamous cell carcinoma cases and 25% of oropharyngeal cancer cases are related to HPV infection (16). Integration of HPV DNA into the cell genome leads to abnormal cell proliferation and malignant progression, which plays an important role in HPV-mediated carcinogenesis (17). Several studies have suggested HPV as a possible cause of breast cancer. However, due to conflicting

Table 2. Association of HPV with clinicopathological features

		HPV negative	HPV positive	Univariant analysis			Multivariant analysis		
				OR	95% confidence interval (CI)	P-value	OR	95% confidence interval (CI)	P-value
Perineural invasion	Yes	27 (29)	2 (28.6)	1.28	(0.26-6.43)	0.762	1.65	(0.26-10.64)	0.596
	No	50 (53.8)	4 (57.1)						
	unknown	16 (17.2)	1 (14.3)						
Lymphovasc ular invasion	Yes	34 (36.6)	3 (42.9)	1.33	(0.24-7.32)	0.74	1.68	(0.21-13.24)	0.621
	No	47 (50.5)	3 (42.9)						
	unknown	12 (13.9)	1 (14.3)						
Surgical margin	Yes	8 (8.6)	0 (0)	1	(0.099-10.51)	1	0.81	(0.04-17.45)	0.891
involvement	No	80 (86)	6 (85.7)						
	unknown	5 (5.4)	1 (14.3)						
Lymph node involvement	Yes	50 (53.8)	3 (42.9)	0.48	(0.1-2.28)	0.354	0.52	(0.09-2.58)	045
	No	33 (35.5)	4 (57.1)						
	unknown	10 (10.8)	0 (0)						
ER expression	No	25 (26.9)	0 (0)	3.35	(0.32-34.66)	0.309	3.01	(0.25-37.02)	0.387
	Yes	57 (61.3)	6 (85.7)						
	unknown	11 (11.8)	1 (14.3)						
PR expression	No	30 (32.3)	3 (42.9)	0.90	(0.19-4.26)	0.891	1.10	(0.13-9.21)	0.927
	Yes	51 (54.8)	4 (57.1)						
	unknown	12 (12.9)	0 (0)						
Ki-67 expression	No	23 (24.7)	2 (28.6)	0.97	(0.18-5.34)	0.972	0.71	(0.10-5.14)	0.736
	Yes	61 (65.6)	5 (71.4)						
	unknown	9 (9.7)	0 (0)						
Tumor grade	well-differentiated	23 (24.7)	0 (0)	1.84	(0.67-5.07)	0.238	1.80	(0.58-5.58)	0.311
	moderately-differentiated	47 (50.5)	5 (71.4)						
	poorly-differentiated	20 (21.5)	2 (28.6)						
	unknown	3 (3.2)	0 (0)						
HER2 expression	No	53 (57)	5 (71.4)	0.62	(0.12-3.32)	0.577	0.57	(0.09-3.49)	0.573
	Equivocal	15 (16.1)	1 (14.3)						
	Yes	13 (14)	1 (14.3)						
	unknown	12 (12.9)	0 (0)						

data on the presence of HPV DNA in tumor samples from breast cancer patients and a lack of clarity about the route of HPV transmission from one organ to another, there are still challenges regarding the role of HPV in breast cancer carcinogenesis (18).

In the current study, the prevalence of HPV in the cancer and control groups was 7% and 1%, respectively and HPVs detected in two groups were of the HPV 16 genotype. Also, in this study, the risk of cancer in HPV positive subjects was almost 5 times higher than in HPV negative subjects, but it was not statistically significant.

Several studies have been conducted in different regions of Iran to investigate the presence of HPV in cancerous and healthy breast tissue. Previous studies reported a prevalence of HPV between 0.6 and 5.7% in the general population in Iran (19, 20). In addition, HPV DNA was detected in various studies from 4 to 86% in breast cancer patients (21). In the study by Khodabandhlou et al. in Iran (2019), the prevalence of HPV in cancer samples was reported as 48.6% and in healthy samples as 16.1%, so the risk of breast cancer was significantly 4.92 times higher in the HPV-positive group than the HPV negative group (22). In the study by Sigaroudi et al. (2011, Iran) reported that the frequency of HPV DNA in breast cancer patients is 25.9% compared to women without cancer (2.4%), and the highest frequency of genotypes in breast cancer patients were HPV types 16 and 18 with an overall prevalence of 53.34%. They also showed that

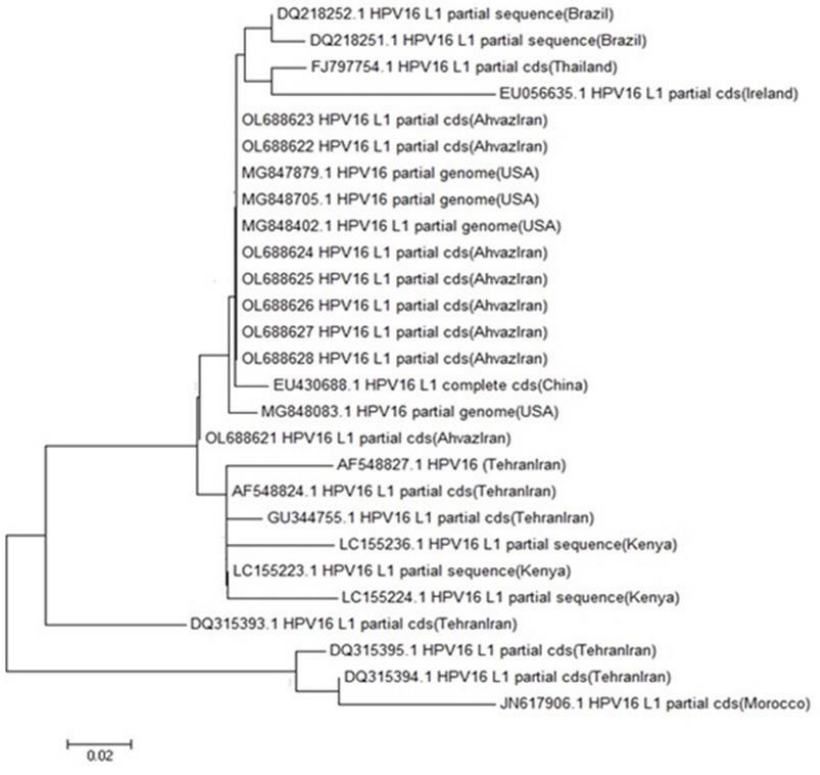

Fig. 2. The Phylogenetic Tree was Constructed by Neighbor Joining for L1 Region of HPV16 Genome Isolated from Ahvaz City. The detected L1 region of isolated HPV from Ahvaz with accession numbers OL688621-OL688628 were compared with different L1 genotypes retrieved from GenBank. The detected L1 HPV genotype 16 isolated from Ahvaz City were clustered with L1 HPV genotype 16 isolated from different regions of world including Brazil, Thailand, USA, Ireland and China The accuracy of the tree was assessed by 1000 bootstrap replicates. The scale bars is equal to 0.05

HPV infection in Iranian women is associated with an increased risk of breast cancer (OR 14.247, 95% CI 1.558-130.284; P = 0.019) (23). A meta-analysis study conducted by Haghshenas et al. confirmed that HPV DNA has a significantly higher prevalence in breast cancer specimens compared to benign breast specimens. In addition, the results showed that high-risk HPV genotypes were more common compared to low-risk HPV (24). In the study by Afshar et al. in Iran, Kerman (2018) reported low HPV prevalence in 8 out of 98 patients (8.2%) in the cancer group and the absence of positive HPV in the control group. In this study, HPV types 16 and 18 were the most common types (62.5%) in positive specimens (25).

A meta-analysis by Simoes et al. of 29 studies from 1990 to 2011 showed an estimated overall prevalence of HPV in breast cancer patients of 23%, ranging from 13% in the European population to 42.9% in African and Australian populations. They also reported an odds ratio of 5.9 for breast cancer in HPV-positive cases compared to controls (26). Other meta-analyses have reported HPV infection as an effective risk factor for breast cancer, reporting high odds ratios ranging from 3.6 to 4.02 (27, 28). Also other studies have reported the different prevalence of HPV in breast cancer patients as follows: 41.6% (2015, Venezuela), 40% (2013, Mexico) and 21% (2008, Japan) (29-31).

Therefore, differential prevalence of HPV in breast tissue has been observed in different geographic areas and even within the same geographic area. HPV genotypes 16 and 18 are the most common virus types found in cancers worldwide (32). However, other types of HPV including 6, 11, 33, 35, 39, 45, 51 and 59 have also been detected in breast cancer patients (33). Two meta-analyses showed a higher prevalence of high-risk HPVs, including 16, 18 and 33, compared to other HPV strains. In a study by Ni Li et al. estimated the frequency of these three high-risk HPVs to be 7.04, 7.13, and 14.36%, respectively, while other HPVs, including low-risk HPVs, were all below 3% (27, 28). In the studies conducted in Mex-

ico, Venezuela and Brazil, the high-risk genotypes HPV-31 and 51 were the dominant genotypes (18, 30, 34). According to the results of the present study and other studies, a wide distribution of genotypes in breast tissue can be seen depending on the geographic region. This controversy and discrepancy in the prevalence and type of HPV genotype can be caused by the different populations studied, insufficient sample size, sexual behavior of the subjects studied and technical issues such as the samples and the quality of the samples, the sensitivity of the techniques used and technical errors (18, 35). Biological evidence has shown that several proteins expressed in high-risk HPV genotypes act as oncoproteins in virus-induced cancer progression. These HPV-related oncoproteins are involved in proliferative processes. In summary, when HPV integrates into the host cell's genome, it begins to express the E6 gene, and this protein inhibits the tumor suppressor protein p53. E7, another HPV-associated oncoprotein, binds to the tumor suppressor retinoblastoma protein. Other proteins like E1 and E2 are also involved in accelerating HPV replication. All of these proteins are accused of damaging and stabilizing DNA and inhibiting tumor suppressor and apoptosis mechanisms and thus tumor development (17). The Salman et al. (2017, England) shows that the high expression levels of E6 and E7 and their interaction with cellular factors can lead to the development of breast tumors. According to several studies, the expression of HPV proteins in breast tissue is a reliable marker for the detection of an active infection. For example, the expression level of E7 in breast cancer tissue has been shown to be higher than in healthy subjects and early-stage cancer patients (36). The mechanism by which HPV can infect mammary gland cells is still unknown. However, some hypotheses have shown that HPV reaches the mammary glands through the mononuclear cells that carry HPV through the lymphatic or blood systems in women with cervical intraepithelial lesions, although some authors conclude that the life cycle of HPV occurs in the layer of epithelial cells, this is impossible. In the second hypothesis, the mammary gland can be infected with HPV through the skin of the nipple, which is a retrograde ductal model of virus transmission. Exposure of the mammary ducts to the outside environment increases the risk of HPV infection because the mammary ducts are open and can serve as an entry point for viral infection. In addition, most breast neoplasms originate from the epithelium of these structures. Some studies suggest that transmission can occur through manual contact between a woman's perineum and mammary gland, which can occur during sexual activity, or through contact of bodily fluids with nipple cervices, which can serve as entry points for HPV (18). In the present study, there was no correlation between HPV status and any of the clinicopathological characteristics. However the chance of ER expression, PR, lymphovascular involvement, perineurial invasion, and higher tumor grade is higher in HPV-positive individuals than in HPV-negative individuals (OR>1), while the chance of lymph node involvement, surgical margin involvement, Ki-67 expression, and of HER2 expression in individuals infected with HPV is less than uninfected individuals (OR<1). No significant association between HPV infection and clinicopathological features of breast cancer was observed in most studies. Antonsson et al. however, showed that HPV-positive breast cancers were smaller early T-stage tumors compared to HPV-negative cancers (37). In the study by Doosti et al. in Iran (2016), consistent with the results of the present study, no significant association was observed between breast cancer tissue pathology and HPV genotype frequency (38).

The limitations of the present study include the small sample size, failure to account for factors affecting DNA quality such as a special extraction kit for paraffin-embedded breast tissue samples and standardized qPCR, which guarantees high detection accuracy, and the lack of an investigation of the relationship between HPV prevalence and other clinicopathological factors and determinants in treatment and prognosis and finally the collection of specimens from the pathology department of a hospital (single center).

According to previous studies and current studies, a statistical population with a large sample size is required to study the relationship between clinicopathological features of breast cancer and HPV infection.

CONCLUSION

In the current study, the prevalence of HPV in the cancer and benign sample was 7% and 1%, respectively and HPVs detected in two groups were of the HPV 16 genotype. Also, HPV infection showed no significant correlation with the clinicopathological features of breast cancer. Based on studies report-

ing the existence of sequences of different high-risk HPV types (oncogenes) in breast cancer tissues, this study confirmed the hypothesis of a possible infectious cause in the development of breast cancer. So far, however, the results have been controversial and inconclusive. Further studies with large sample sizes are needed to demonstrate the link between HPV and breast cancer.

ACKNOWLEDGEMENTS

This research was carried out by Ali Ramezani PhD (virology) student with registration number OG-9809 and was supported by Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

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