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Original Article

Association of two high-risk strains of human papillomavirus (HPV18 and HPV16) with breast cancer in the patients using polymerase chain reaction

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Abstract

Introduction: Two high-risk strains of human papillomavirus (HPV18 and HPV16) immortalize human mammary epithelial cells and reduce their growth factor requirements. Recent studies have identified HPV in a broad range of breast cancer samples. Therefore, the study aimed to assess the association of the mentioned high-risk HPV strains (HPV18, HPV16) with breast cancer in the patients using polymerase chain reaction (PCR) due to the increasing prevalence of breast cancer.

Methods: The current work was a retrospective study involving 60 participants (40 patients with breast cancer and 20 healthy people) at Tabriz University of Medical Sciences in 2018-2019. The participants were selected using a convenience sampling method. PCR was used to detect DNA genomes of HPV18 and HPV16. Data was analyzed using Phi and Cramér's V with SPSS v. 21 by taking into account OR = 95% and $P = 0.05$.

Results: There was a significant relationship (P value = 0.014) between HPV18 and HPV16 infection and breast cancer.

Conclusion: A significant relationship was found between the presence of DNA genomes of HPV16 and HPV18 and breast cancer. Therefore, HPV has a significant role in breast cancer.

Introduction

Breast cancer is one of the most important health problems worldwide. It is one of the leading causes of cancer death in women.[1] Although numerous risk factors linked to breast cancer were identified so far, the precise causes of the disease are unclear. This triggered a number of studies for identification of new factors that might be the cause of this disease.[2]

The known risk factors for breast cancer include age, family history, use of oral contraceptives, alcohol consumption, genetic and epigenetic factors (e.g. mutations in BRCA1, BRCA2, and TP53 genes), cumulative exposure of breast epithelium to elevated levels of steroid sex hormones, and lifestyle.[3]

Viral infection also raised so many discussions about the cause of breast cancer in numerous studies. Recent studies have assessed the association of viral infections (e.g. Epstein-Barr virus [EBV], human papillomavirus [HPV], and mouse mammary tumor virus) with breast cancer.[4]

A study showed that the herpes simplex virus (especially HSV1) could act as a cofactor in oncogenesis of breast cancer. Some studies have shown that these viruses play essential roles in the initiation of breast cancer. HPV has a small double-stranded DNA genome. It belongs to the family Papillomaviridae.[5] More than 100 HPV genotypes are identified so far. Based on infected tissue, papillomaviruses are classified into cutaneous, mucosal, and genital types.[6]

Although nearly 90% of HPV infections are asymptomatic and the immune system spontaneously clears the detected infections within 1–2 years, sometimes the infection can cause cancer when associated with other factors.[7] For example, 90% of cervical infections caused by HPV18 and HPV16 may eventually lead to cervical cancer. Further assessment of the association of HPV with breast cancer has shown that high-risk strains of HPV (HPV18, HPV16) immortalize human mammary epithelial cells and reduce their growth factor requirements.[8] Recent studies have identified HPV in a broad range of breast cancer samples. Therefore, the study aimed to assess the association of high-risk HPV strains (HPV18 and HPV16) with breast cancer for patients using polymerase chain reaction (PCR) due to the increasing prevalence of breast cancer.

Materials and Methods

Study design

The current work was a retrospective study involving 60 participants aged 23 to 70 years old (40 formalin-fixed

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paraffin-embedded blocks from breast cancer patients and 20 tissue samples from healthy people) at Tabriz University of Medical Sciences in 2018-2019. Samples were selected using a convenience sampling method. Considering the financial resources and the limited number of samples minimum sample size was estimated as 60 (P value = 0.7 and confidence interval [CI] = 95%). Among the selected samples, 40 samples were collected from malignant tissues and 20 (control) from benign (fibroadenoma) tissues. Inclusion criteria were healthy people in the control group and patients with breast cancer in the case group. Exclusion criteria were the history of other types of cancer, interventional therapies, smoking, opioid abuse, and alcohol consumption.

Method

To extract DNA genome, formalin-fixed paraffin-embedded blocks were cut using a microtome (made in England) to generate six to ten thin sections of tissue with 7-micrometer thickness. The sections were poured into DNase/RNase-free microtubes (1.5 mL). After deparaffinization, xylene (made in Germany) was removed with 100% ethanol and then DNA was extracted using salting out technique. Lysis buffer (Tris, NaCl), EDTA, sodium dodecyl sulfate, protease K (made in Germany), NaCl (5 M), cold isopropanol, 70% ethanol, and TE buffer were used in PCR followed by multiple rounds of centrifugation at 14000 rpm and hot water bath. DNA extract concentration and purity were determined by NanoDrop (made in the USA). The ratio of optical density was measured at 260 to 280 nm. The DNA samples whose ratios were between 1.8 and 2 were chosen for PCR with specific beta-actin gene primers.

PCR for amplification of beta-actin gene

PCR was used to amplify beta-actin gene of tumor samples with the help of predesigned primers listed in Table 1. The total reaction volume was 25 µL and 0.2 mL tubes were used. Mastermix (12.5 µL) (made in Denmark), distilled water (10.5 µL), extracted DNA as the template DNA (1 µL), forward primer (0.5 µL), and reverse primer (0.5 µL) were used for PCR. The steps to PCR were initial denaturation at 94°C for five minutes, thirty cycles of denaturation at 95°C for one minute, primer annealing at 61°C for 50 seconds, initial extension at 72°C for 45 seconds, and final extension at 72°C for 10 minutes. PCR products were separated by electrophoresis on a 1.5% agarose gel to detect 161-bp band.

PCR for identification of HPV18 and HPV16 genes

After calibration of HPV18 and HPV16 DNA tests and deign of specific primers for these viruses as shown in Table 1, PCR was performed again for the samples positive for beta-actin gene in order to detect and identify HPV18 and HPV16 DNA genomes. PCR consisted of initial denaturation at 94°C for 10 minutes, 35 cycles of denaturation at 94°C for one minute, primer annealing at 54.8°C for HPV16 and 55°C for HPV18 for one minute, extension at 72°C for one minute, and final extension at 72°C for 5 minutes. PCR products were separated by electrophoresis on agarose gel 5.5% to detect 120-bp band for presence of HPV16 and 412-bp band for presence of HPV18.

Statistical analysis

Collected data was analyzed using SPSS. Cramér's V and Phi were used to determine strength of the association and 95% CI was considered for the odds ratio. Significance level was $P < 0.05$

Results

Extraction and quality assessment of DNA

The optical density ratios of all extracted DNA were calculated by NanoDrop and ranged from 1.8 to 2. Beta-actin genome was amplified the by PCR and 161-bp was detected on the agarose gel.

Presence of HPV18 and HPV16 in malignant and healthy breast tissue samples

DNA from breast cancer patients and normal controls were amplified using specific primers designed for HPV18 and HPV16. PCR products were separated by electrophoresis. Findings showed 25 positive results for HPV16, 10 positive results for HPV18, four positive results for HPV18 and HPV16, and 20 negative controls (Table 2).

Cramer's V coefficients for HPV16 and HPV18 virus were 0.598 and 0.316 respectively. Since the significance level was less than 0.5, the null hypothesis was rejected and a significant relationship was found between infection with HPV18 and HPV16 and breast cancer (0.014). The odds ratio with 95% CI for HPV16 and HPV18 were 43.125 (4.177 - 278.818) and 7.54 (0.894 - 62.126), respectively. Since this ratio was not one, the two factors depend on each other and HPV infection increases the risk of breast cancer.

Table 1. Beta-actin gene of tumor samples

Dedication	Initiator sequence)3'to5')	Product size
Beta actin	AGACGCAGGATGGCATGGG GAGACCTTCAACACCCCAGCC	161
HPV16	GAGACCTTCAACACCCCAGCC R: CGT GTT CTT GAT GAT CTG CA	120
HPV18	ATAGCAATTTTGATTTGTC AAACTCATTCCAAAATATG	415

HPV, Human papillomavirus.

Table 2. Presence of DNA genomes of HPV18 and HPV16 in cancer and healthy samples

Type of virus	Non-cancerous	Cancerous	*P* value
HPV16	-	25	0
HPV18	-	10	0.014

HPV, human papillomavirus.

Discussion

Breast cancer is a lethal disease and the second leading cause of cancer death in women. Nearly one in eight women is diagnosed with breast cancer in developed countries. Stages of the disease range from early stages to advanced stages. Therapies in most cases include complete removal of breast tissue, chemotherapy, radiotherapy, and hormonal therapy. Global studies have warned against the increasing prevalence of the disease in different countries.[9] Scientists have mentioned several factors accounting for less than half of the breast cancer cases. Molecular mechanisms of breast cancer are unknown and no adequate information is found in this regard. Viral infection is one of the important risk factors for breast cancer. It was widely studied and numerous conflicting results were found. Some confirmed and others rejected this association. Therefore, an association of HPV with breast cancer is still a controversial issue.[6]

For the first time, Bittner suggested viruses as one of the factors leading to breast cancer. They identified an unknown factor in the milk of mice that later caused breast cancer in growing baby mice. The unknown factor later identified as mouse mammary tumor virus.[10]

Di Lonardo et al discussed the relationship of HPV with breast cancer for the first time in 1992. They identified HPV16 DNA in 30% of breast cancer samples. Later, Di Lonardo et al reported EBV in breast cancer samples. Viruses (e.g. HBV, EBV, and HPV) are reported to cause genetic instability, uncontrolled cell proliferation, expression of oncogenes, inhibition of apoptosis, and immortalization of cancer cells. Oncogenic viruses were reported as risk factors for one-fifth of cancer cases. HPV was assessed in patients with breast cancer in this study.[11]

This high-risk virus synthesizes E6 and E7 oncoproteins, which bind to retinoblastoma, and block Rb/E2F binding. HPV18 is the most prevalent type of HPV identified in breast cancer cells. HPV16. HPV6, HPV11, and HPV23 were also determined in breast cancer cells. In this study DNA of HPV16 and HPV18 were identified in 40 and 10 malignant tissues, respectively.[7] These results were consistent with the results of the study by Seyedi Alavi et al[12] who assessed 50 cases of breast cancer and identified HPV DNA in 50% of breast carcinomas. Half of these cases were caused by high-risk HPV. Fifty-four patients with breast cancer were assessed in another study and HPV DNA was identified in 50% of the samples. Salman et al examined 79 patients with breast cancer in north of Iran. A quarter of the samples were positive for HPV16 and HPV18.[13] Salman et al examined 110 patients with breast cancer in 2017 and identified HPV in 42% of the cases. Of these, 12% were positive for HPV18. A review of 37 different papers assessing the association of HPV with breast cancer using different molecular methods (e.g. PCR) showed that six papers confirmed this association, five papers rejected this association, and definitive results were not found in 26 papers. HPV prevalence was reported from 0 to 82% in different samples. Nevertheless, it is controversial whether HPV infection is a risk factor for breast cancer.[13] Many researchers claimed that failure to identify HPV, different methods used to identify HPV and different analysis methods might be the cause of these confounding results. Another reason for these contradictory results might be assessment of association of one type of virus with breast cancer in each study and various types of viruses were not assessed in a single study.[7,8,14]

Limitations

The only limitation of this study was small sample size due to high cost of laboratory tests.

Recommendations

It is recommended that health systems screen women at high risk of breast cancer for HPV. It is also recommended to vaccinate high-risk populations given the increasing production of HPV vaccine.

Conclusion

This study determined a significant relationship between HPV16, HPV18 and breast cancer. The association of HPV DNA with breast cancer can be assessed using molecular methods along with other methods in statistical population of different provinces of Iran with different ethnic parameters to identify high-risk population given the contradictory findings regarding this association in the world and in Iran.

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Authors' Contribution

All authors contributed to conception and design of the study and literature review, collected the data and contributed to data interpretation and drafting the manuscript.

Ethical Approval

This research was approved by regional ethic committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1397.598). Informed written consent forms were collected from the participants. Necessary arrangements were made with authorities of chemotherapy and radiotherapy clinics of Ghazi Tabatabai and Shahid Madani hospitals. All tests were free of charge. The participants volunteered to participate in the study.

Study Highlights

What is current knowledge?

- There is an association between dangerous strains of papilloma viruses and breast cancer

What is new here?

- Both HPV18 and HPV16 play a role in breast cancer

Conflict of Interest

Authors declare no conflict of interest in this study.

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