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Original Article

Frequency of Human Papillomavirus Genotypes 6, 11, 16, 18 And 31 in Paraffin-Embedded Tissue Samples of Invasive Breast Carcinoma, North- East of Iran

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ABSTRACT

Background &Objective: **Breast cancer is the most common female malignancy. Detection of DNA of human papillomaviruses (HPVs) in breast carcinomas suggests that the virus may play a role in the pathogenesis of this disease. The aim of this study was to evaluate the frequency of HPVs genotypes 6, 11, 16, 18 and 31 in paraffin-embedded tissue samples of invasive breast carcinomas.**
Methods: **Three hundred and twenty six paraffin-embedded tissue samples of breast cancer were studied. PCR was performed using specific primers for HPV genotypes.**
Results: **Of total 206 (63.2%) samples positive for Beta-globin gene, 54 (26.2%) were HPV-positive and 152 (73.8%) were negative for HPV. Distribution of HPV genotypes were as follows: 19 (25.7%) were positive for genotype 11, 5 (6.8%) were positive for genotype 6; and 2 cases (2.7%) were positive for both genotypes 6 and 11. Samples were also screened for HPV genotypes 16, 18 and 31 but none was positive.**
Conclusion: **The current study confirmed the association of HPV and breast cancer. However, all samples were negative for high-risk HPV types 16, 18 and 31.**

Keywords: **Human Papillomavirus (HPV); Breast Cancer; Polymerase Chain Reaction (PCR)**

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Introduction

Human Papillomavirus (HPVs) can be transmitted between individuals through genitals, anal, mouth or breast (1). Breast cancer is the most common malignant tumor among women and the second highest cause of cancer-related death (2,3). Among Iranian women, mortality due to breast cancer is higher than deaths due to other kinds of cancer (4). More than 1,000,000 new cases are diagnosed each year in the United States of America and it caused about 458,000 deaths worldwide alone in 2008(5). Although the incidence of breast cancer has almost doubled in the past four years in the United States, the death rate remained noticeably constant. The loss of cellular regulation that gives rise to most of cancers is due to genetic damages. Numerous factors, especially mutations, are considered the risk factors in the onset of cancer (6,7). These are often unknown mutations, which are inherited or acquired. Breast cancer is rare in people under 25 years unless a family history of breast cancer exist (8). The risk of breast cancer increases with age. Although there are many patients without a family history of breast cancer, 13% of women with a family history are found to develop breast cancer. This finding reinforces the probability of the involvement of other non-genetical factors (9,10). Exercising, using medication, breastfeeding, life style, stress, genetic aptitude for the disease and other factors may have role in the genesis of breast cancer (9, 11,12). Virus infection may be responsible for breast cancer. However, more studies are required to confirm their associations.

HPVs are small and are capable of causing warts in some vertebrates, including humans. Based on phylogenetic relationships and their role in benign and malignant cervical cancers, HPVs are classified into three categories: high-risk, medium-risk and low-risk. HPV types 16, 18 and 45 fall into high-risk category while intermediate-risk group includes HPV types 31, 33, 35, 39, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82. Low-risk types include types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81. These viruses have the ability to cause cancer in humans and animals (13). Some HPVs cause cervical cancer and epithelial tumors (14-17).

In the present study, the authors evaluated 326 paraffin-embedded samples in order to identify possible relationships between HPV infection and manifestations and progressions of breast cancer.

Materials and Methods

Sample collection

After obtaining approval from ethics committee of Mashhad University of Medical Sciences, samples collected between August 2002 and September 2012 from pathology departments of Imam Reza and Qaem hospitals in the city of Mashhad, northeast of Iran were studied in a cross-sectional study. Three hundred and twenty six paraffin-embedded samples of breast carcinoma were chosen and confirmed pathologically. Five sections of 10-20 micron were extracted from paraffin blocks in sterile conditions.

DNA extraction and PCR

Zylol-Ethanol method was used for deparaffinization of samples. For this purpose, paraffin-embedded samples were sliced in diameter of 20 micrometers using microtome and were kept in 1.5 ml micro-tubes. In the next step, 1 ml of zylol was added and the mixture was shaken for 30 min at 25 °C. Following that, it was centrifuged at 13000 rpm for 10 min. The supernatant was removed. This step was repeated for several times. Five hundred microliters of absolute ethanol was added to the pellet and it was shaken and centrifuged at 13000 rpm for 10 min. In the final step, the supernatant was removed again and this step was repeated for the

second time. The pellet was kept at 25 °C so that it could lose ethanol but it was not allowed to dry completely. PCR primers were designed by Gene Runner (Hastings Software Inc.) software according to the pattern sequences in GenBank.

β-Globin gene PCR to assess the quality of extracted DNA

The quality of the extracted DNA from paraffin-embedded tissue samples was assessed by primers of β-globulin gene. PC04 and GH20 primers were used, which gave rise to PCR products of 260 bp in length (Table 1).

Table 1: Primer sequences and the corresponding PCR product of Beta globin and L1

Gene	Primers		Product size(bp)	Reference
Beta globin	GH20	GAAGAGCCAAGGACAGGTAC	260	16
	PC04	CAACTTCATCCACGTTCACC		
L1 (HPV)	+ GP6	GAAAAATAAACTGTAAATCATATTC	142	18
	+ GP5	TTTGTTACTGTGGTAGATACTAC		

In order to amplify beta globin gene, the reaction mixture contained 0.8 µl of DNA, 0.4 µl of dNTPs (10mM), 0.8 µl of Taq DNA polymerase (5 U/µl), 1.6 µl of MgCl2 (25mM) , 10 pmol/µl of each primer, and 12.2 µl of H2O (DNase-RNase free). PCR program was 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 45 s and a final extension of 72 C for 5 min.

PCR-based method for HPV genotyping

Genotyping was performed by PCR using virus-specific primers for different subtypes (Table 2). The reaction mixture for PCR reaction of HPV types 6, 16, 18 and 31 contained 2 µl of DNA, 1x reaction buffer, 10 µmol dNTPs, 50 mmol MgCl2, 10 pmol of each primers and 5 u/µl Taq DNA polymerase (Cinnagen, Iran). However, for type 11, 25mmol MgCl2 and 3 µl of DNA was used. PCR program was 94 °C for 5 min; 35 cycles including 94 °C for 45 s; Melting temperatures of 55 °C for type 11, 57 °C for type 6, 60.8 °C for types 18 and 31, and 63 °C for type 16; 72 °C for 45 s and was followed by final extension of 72 °C for 10 min. It produced the PCR products of 360 bp for type 11, 280 bp for type 6, 420 bp for types 18 and 31, and 400 bp for type 16. Finally, PCR products were visualized on a 2% agarose gel. Using specific primers, samples, which produced the corresponding fragments, were considered positive for that type of HPV.

Table 2: Sequence of genotype-specific primers used in the PCR

Primer	Sequence	Genotype	Reference
HPV 6 F	TAG TGG GCC TAT GGC TCGTC	6	(17)
HPV 6 R	TCC ATT AGC CTC CAC GGG TG		
HPV 11 F	GGA ATA CAT GCG CCA TGT GG	11	(17)
HPV 11 R	CGA GCA GAC GTC CGT CCT CG		
HPV 16- E6 F	CAG GAC CCA CAG GAG CGA CC	16	(18)
HPV 16 –E6 R	ATC GAC CGG TCC ACC GAC CC		
HPV 18 –E6 F	GCT TTG AGG ATC CAA CAC GG	18	(18)
HPV18 – E6 R	TGC AGC ACG AAT GGC ACT GG		
HPV 31-E6/E7 F	GAA ATT GCA TGA ACT AAG CTC G	31	(18)
HPV 31-E6/E7 R	CAC ATA TAC CTT TGT TTG TCA A		

Data Analysis

Frequency charts and diagrams together with SPSS v.20 (Chicago, IL, USA) was used for data explanation purposes.

Results

Results of beta-globin

Overall, 326 samples were used. Age ranged between 24 and 74 years. The maximum age of positive samples was between 35 and 40 years. Samples from 326 patients with invasive breast carcinoma showed that 206 cases (63.2%) were positive for PCR of Beta-globin gene and 120 cases (36.8%) were negative. Among positive samples, 54 cases (26.2%) were positive for HPV and 152 cases (73.8%) were negative. While among Beta globin-negative samples, 20 cases (16.7%) were HPV-positive and 100 (83.3%) were negative. Totally, 74 out of 326 samples (22.7%) were positive for HPV and 252 cases (77.3%) were HPV-negative.

Distribution of HPV genotypes

Nineteen cases (25.7%) were positive for genotype 11, and 5 cases (6.8%) were positive for genotype 6. Two cases (2.7%) were found to be positive for both genotypes 6 and 11 (Fig.1). Samples were also screened for genotypes 16, 18 and 31 and showed no positive result. Genotype of the virus was unknown for 48 cases (64.9%). In addition, there were 5 beta-globin negative cases among the 19 patients with genotype 11, and one out of five patients who was positive for genotype 6, was negative for beta-globin.

Distribution based on tumor grade in HPV positive specimens

Among all HPV-positive cases, 1.4% had Grade A, 8.1% had grade B, 10.8% had grade C and 79.7% had unknown grade.

Distribution according to involvement of axillary lymph node

Of total HPV-positive samples, 16.2% had axillary lymph node involvement, 1.4% had no involvement and for the remaining 82.4%, the involvement was unclear.

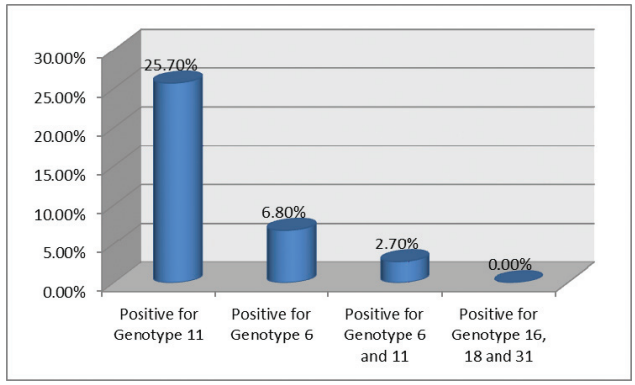

Fig. 1: Number of patients according to HPV genotypes

Discussion

Breast cancer is the most common malignant tumor in women. HPV is a small, non-enveloped virus containing DNA, which is highly specific for both species and tissue. Several studies supported the role of HPV in breast cancer. Seyed Alavi and colleagues studied the role of HPV infection in breast cancer and its correlation with clinical parameters. The role of HPV in 24 cases (48%) of the total cases of breast cancer was identified. They also showed that 13 of them (26%) were infected with high-risk types of whereas in 8 cases (16%), they were infected with low-risk types of HPVs. In three breast cancer tissues (6%), both types of HPVs (High-risk and low-risk) were detected (3). Widschwendter et al. studied 11 patients with simultaneous cervical and breast cancers and observed that seven cases were positive for HPV (18). Kan et al. performed a study on DNA sequences of HPVs involved in breast cancers to assess possible correlations. They demonstrated that 24 samples (48%) of total 50 samples were positive for HPV (19). Khan and colleagues studied the association of HPV in breast carcinoma in Japan. In 26 patients (21%), breast carcinomas were observed. Among them,

HPV type 16 was the most prevalent genotype, which has been considered as the main culprit (92%) for manifestation of disease (20). HPV DNA is highly associated with breast cancer (21, 22). Yasmeen and colleagues conducted a study to evaluate the role of onco-proteins E6 and E7 of HPV type 16 in cell invasion and metastasis of breast cancer. They reported that E6/E7 of genotype 16 is capable of making two invasive and metastatic cell lines: BT20 and MCF7 (23). HPV infection may have a significant role in the development of breast cancer (20, 24-27). Lee and colleagues used PCR method to study the role of HPV DNA in breast carcinoma. 24.49% of the cases of breast carcinomas were associated with HPV virus. In addition they also detected 40 types of HPVs with HPV type 33, 18, 16 and 35 being the most prevalent ones (28).

Some studies, on the other hand, did not provide evidence in favor of the role of HPV in breast cancer. Wrede et al. and Gopalkrishna et al. conducted studies to evaluate the presence of HPV types 16 and 18 in breast carcinomas. However, none of the samples showed any correlations (29, 30). A study was conducted to investigate the presence of HPV DNA in breast carcinoma in Korean women and the relationship between HPV and breast cancer progression. However, no noticeable correlations were observed (31). Throughout the last decade, several independent studies in different regions reported no significant correlation between HPV and the incidence and progression of breast cancer (32-35). The role of HPV in cancers such as uterine cervix has been widely accepted.

Conclusion

The current study confirmed the role of HPVs in breast carcinoma among embedded tissue samples of invasive breast carcinoma in northeast of Iran. However, further investigations with a much larger sample size are required to validate the exact role of HPVs in the prevalence and progression of this cancer.

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Conflict of interest statement

The authors declare that there is no conflict of interests.

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