Low Prevalence of Human Papilloma Virus in Patients with Breast Cancer, Kerman; Iran

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Abstract

Breast cancer is the first of the most important causes of the deaths of women in the world and in Iran. There are various causes and causes of this cancer, one of which has recently been described as a cause of breast cancer, is the human papillomavirus (HPV). The HPV is transmitted through sexual contact and skin lesions. There are more than 100 types of HPV that can influence different parts of the body. Some types of HPV can cause cancer (such as cervical or anal cancer) and others can cause warts (such as genital or plantar warts). To study the risk of HPV infection in Breast Cancer, we managed a Case-Control study in Kerman, southeast of Iran. For this purpose, 98 paraffin blocks of breast cancer and 40 paraffin blocks of fibrocystic as a control were tested for the presence of HPV DNA using Real-Time PCR, and HPV typing was done using INNo-Lippa assay. HPV DNA was detected in 8 out of 98 patients (8.2%), while it was not detected in the control group samples. HPV types 16, 18 were the most common (62.5%) types in positive samples. The prevalence of HPV in patients with breast cancer of Iran is very low and less than other regions of the world, it seems that maybe rout of transmission of HPV in Iran is under control. No one knows exactly why breast cancer occurs. The environment, hormones, Viruses, or your lifestyle could all play a role in the development of breast cancer. Currently, Vaccination is the best way to prevent cancer that’s due to HPV. However, additional studies on the larger group of patients are needed to explain the roles of HPV in Breast cancer.

Keywords: Breast cancer- human papilloma virus- Kerman- Iran

Introduction

Breast cancer is one of the most common malignancies and the most common cause of deaths among the women around worldwide. This is known to be the second deadliest cancer among American women and the first deadly cancer among European women (Balekouzou et al., 2016). In Iran, breast cancer is ranked among the first cancers Known in women (Kolahdoozan et al., 2010). Breast cancer is an out-of-control change in epithelial cells in the breast tissue, with abnormal growth in the production glands, lobules, or ducts that connect lobules to the nipple, and clinically diagnosed as a heterozygous disease, mainly due to a wide variety of genetic changes, such as genomic rearrangement, mutation, and the acquisition or loss of the chromosome (Fard, 2012; Boudersa et al., 2017). Several factors contribute to the incidence of breast cancer, including age, gender, family history, genetic disorders, physical inactivity, alcohol consumption, tobacco and Oral contraceptives and viruses that they are intracellular microorganisms (Akil et al., 2008; Aguayo et al., 2011). Recently, evidence has shown that some viruses, especially Human Papilloma Virus (HPV), may play a role in the progression and making breast cancer, as well as MMTV (Mouse Mammary Tumor Virus) and EBV (Epstein Barr Virus) viruses from the first candidate as the cause of breast cancer (Malekpour Afshar et al., 2015). Breast cancer is the second most common cancer after lung cancer (1.8% in both sexes, 12.9% of the total) with an estimated 1.7 million cases (11.9%) in 2012 worldwide. In terms of mortality, the fifth rank after lung cancer (1.6 million), the liver (745,000), the stomach (723,000), the large intestine (694,000) with 522,000 worldwide (Malvia et al., 2017). According to Cancer Research UK, the UK incidence rate in 2013 was the sixth highest in Europe with 53 300 new breast cancer cases diagnosed in women (Mendizabal-Ruiz et al., 2009). Papilloma is a human virus belonging to the papilloma family, which is in the alphavirus genus. HPV genome codes seven primary proteins E1 to E7 and two delayed proteins L1 and L2 (Sigaroodi et al., 2012). The HPVs belong to a large family of common viruses that infect cutaneous and mucosal epithelial surfaces (skin, genital) and cause both benign and malignant hyper proliferative lesions (Afshar et al., 2013). HPV can be transmitted through the genitals, anal, mouth or...
breastfeeding. HPV is associated with diseases such as cervical cancer, anal cancer, head and neck cancers, and some other cancers such as breast and lung cancer have been proven (Chang et al., 2012; Balekouzou et al., 2016). Although about 90 percent of HPV infections are asymptomatic and usually cleared by the immune system in two years, but after a long delay, HPV sustainability can lead to malignancy in the presence of appropriate risk factors. Cervical infection with high-risk HPV types 16 and 18 causes 90% of cervical cancers (Damin et al., 2004; Duo et al., 2008). Approximately 200 human papillomaviruses types are known, HPV causes a wide range of epithelial hyperplasia lesions to be classified into mucosal and cutaneous lesions. Two major proteins in High risk HPV (E6 and E7) have the ability to make primary epithelial cells of the breast to malignant cells, and evidence has emerged about the potential role of this virus in breast cancer production (Dilonardo et al., 1992; Kroupis et al., 2006; Haghshehas et al., 2016). Clinical studies have controversial information on the presence of HPV in breast cancer, which is in Europe HPV16 and 18, and in women of Japan and China, HPV33 is associated with breast cancer. The E7 and E6 proteins of High risk HPV express in cancers such as cervical, colon, and deactivate two suppressor proteins, retinoblastoma protein (PRb) and p53, respectively (Trimeche et al., 2007; Yahyapour et al., 2013; Mukkamalla et al., 2016; Yan et al., 2016). E6 protein helps to destroy the P53 by binding to the E6-AP subunit, a component of the proteolysis pathway of ubiquitin, and the E7 protein binds to PRb as well as other small proteins, such as P130 and P107, which can disrupt the cell cycle (Zhou et al., 2015). To date, studies on the role of HPV in breast carcinogenesis have generated considerable controversy, DiLonardo et al.; were the first to report the positive relationship between HPV and breast cancer, by demonstrating the presence of HPV in 29% of breast carcinomas (Dilonardo et al., 1992). Despite breast cancer as one of major public health in the IRAN, studies on the etiology of breast cancer needs to further attention. Given that HPV infection may be a possible risk factor in the development of the breast cancer which causes high mortality among the global female population, and in the IRAN, there are no studies, to our knowledge, that investigates the implication of HPV infection in breast cancer development (Afshar et al., 2013; Malekpour Afshar et al., 2015). Therefore, this study with aims to determine the presence HPV and HPV genotypes in breast tissues from patients with breast cancer in Kerman population (Southeast of IRAN) using highly sensitive molecular methods.

Material and Methods

Study subjects

Samples were provided with 98 paraffin blocks of breast cancer and 98 paraffin blocks of non-breast cancer as a control (fibrocystic) during August 2015 to September 2016 in the pathology department of Bahonar Hospital in Kerman. Paraffin embedded blocks were processed using xylene for remove of paraffin that possibility of isolating DNA for HPV detection assays. This method has received approval for clinical use from the U.S. Food and Drug Administration. The personal consent has been obtained from the cases and controls.

Deparaffination samples

Paraffinized blocks from the 98 tumor samples and 40 no tumor samples were cut in 3-μm sections and 5 sections, patients were collected in the same micro-centrifuge tube. Samples were de-waxed in 800 μl xylene; all micro-centrifuge tube located about 10 min in a 60°C heated block and centrifuged at 10,000 rpm for 1 minute, the supernatant was removed. This step was then repeated three times. Add 500 μl absolute ethanol, centrifuged at 10,000 rpm for 1 minute, the samples were then dried in a 70°C heater block with open lids for 5-10 min for remove residual ethanol.

Tissue digestion

According to samples (biopsy or Paraffinated blocks), 200-400 μl of Tissue Lysis Buffer was added to each tube [4 M Urea, 200 mMTRis, 20 mMNaCl, 200 mM EDTA; PH=7.4 (25°C)]. To all tubes added 20-40 μl proteinase K, Samples were gently vortexes and located about 10 min in a 60°C heater block, and all samples were subsequently incubated at 37°C overnight.

DNA Extraction

The next day, 200 μl of Binding Buffer [6 M Guanidine- Hcl, 10mM Urea, 10mM Tris-Hcl, 20% Tritonx-100 (v/v); PH=4.4 (25°C)] was added to each tube with gently vortex. DNA was isolated using a QIAnamp DNA Mini kit (Qiagen, Germany) according to the manufacturer’s instructions. Extracted DNA pellets were suspended in 70μl of pre-warmed Eulution buffer and stored at -70°C until use.

Qualitative Real Time PCR

After DNA extraction, for detection and screening positive samples with HPV, a qualitative Real time PCR based on SYBR Green was done. The primers used in this study were general primers from MY09 and MY011 pairs (MY09: 5'-CGT CCM AAR GGA WAC TGA TC-3' and MY011: 5'-GCM CAG GGW CAT AAY AAT GG-3').

INNO-LiPA HPV detection and genotyping

After performing a PCR test and identifying positive samples, the Inno-lippa test was performed to identify the types of HPV. The INNO-LiPA® HPV Genotyping Extra II (Fujirebio Diagnostics, Sweden) kit was used for this experiment, the steps of which have been described previously in detail (Afshar et al., 2013).

Statistical analysis

For statistical analysis, a Chi square test was performed to assess the independence of the variables, with the IBM SPSS Statistics software, version 20. Values less than or equal to 0.05 were considered statistically significant.

Results

In this study, 98 cases of breast cancer tissue
with a mean age of 48.09±3.5 years and 98 non-breast cancer tissue (fibrocystic) with an average age of 41.22±3.48 years old age, were investigated. Tumor grades were classified to three groups; High grade, Intermediate, Tubular and Low grade. Distribution frequency of tumor grades was shown in Figure 1. After HPV real time PCR, out of 98 tumor samples 8 (8.2%) samples were positive for HPV infection, in control group there were not any HPV positive. The mean age of years in positive samples were 49.53±4.1. For HPV typing, the Inno-Lippa was done, HPV types 16,18 in 5 cases (62.5%) and HPV types 31,33 in 3 cases (37.5%) were positive, frequency of other HPV types were shown in Figure 2. Out of 22 high grade tumor samples, HPV were positive in 4 samples (18.18%), in Tubular types all three patients were negative for HPV and in other degrees of tumors was shown in Figure 3. Following Pearson Chi-Square

Considering the P value obtained (P = 0.051), which is equal to 0.05, there is a statistically significant relationship between HPV and tumor type, a relation between breast cancer and HPV. Human papillomavirus is most common in patients with the age group of 40-60 years old age (with an average age of 49.83), there are 55 patients that were four samples positive for HPV with 56.1%, while in patients with an age range of 60-80 (with an average age of 66.25) there are 16 patients only 2 samples were positive with the lowest frequency of 16.3%. Regarding Chi-square test, the P value (P. Value = 0.787), which is greater than 0.05, there is no significant relationship between HPV and the age of breast cancer (Table1). Human Papilloma Virus has most frequent in lymph nodes not involved with the tumor. According to statistical analysis, there was no significant relationship between the frequency of HPV in tissue samples of breast cancer and tumor lymph nodes

Figure 1. Distribution Percent Frequency of Tumor Grades

Table 1. Age Group Distribution in HPV Positive and HPV Negative

| Age Group | HPV positive (%) | HPV Negative (%) | Total (%) | P.Value |
|-----------|-----------------|-----------------|-----------|---------|
| 20-40     | 2 (7.4)         | 25 (92.6)       | 27 (27.5) |         |
| 40-60     | 4 (7.3)         | 51 (92.7)       | 55 (92.7) | 0.787*  |
| 60-80     | 2 (12.5)        | 14 (87.5)       | 16 (16.3) |         |
| Total     | 8 (8.2)         | 90 (91.8)       | 98 (100)  |         |

* Pearson Chi-Square

Table 2. Summary of Studies on Breast Cancer and HPV Diagnosis and Typing

| Year | Author                  | Location  | N   | HPV positive | Method, Primer type       | References |
|------|-------------------------|-----------|-----|--------------|---------------------------|------------|
| 2005 | C-Y Kan                 | Australia | 50  | 24 (48%)     | PCR(E6)                   | (Kan et al., 2005) |
| 2004 | Damin AP                | Brazil    | 101 | 25 (24.75%)  | PCR (E6)                  | (Damin et al., 2004) |
| 2008 | N A Khan                | Japan     | 124 | 26 (21%)     | PCR(E6), Inno-Lippa       | (Khan et al., 2008) |
| 2008 | Akil N                  | Syria     | 113 | 69 (61.06%)  | PCR(E1) and microarray    | (Akil et al., 2008) |
| 2017 | Silvia Delgado-Garcia   | Spanish   | 437 | 251 (51.8%)  | PCR (GP5/GP6, CLART® and DIRECT FLOW CHIP®) | (Delgado-Garcia et al., 2017) |
| 2017 | Salman NA               | London, UK.| 110 | 46 (42%)     | PCR(L1), HPV- HCR Genotype-Eph kit | (Salman et al., 2017) |
| 2005 | Ethel-Michele de Villiers| Germany  | 29  | 25 (80%)     | PCR(L1),RS42 and KM29     | (de Villiers et al., 2005) |
| 2015 | Fernandes A             | Venezuela | 24  | 41.67%       | INNO-LIPA genotyping extra kit | (Fernandes et al., 2015) |
| 2012 | Simões PW               | systematic review | 2211 | 23.0% | PCR(GP5/GP6,L1,E6), | (Simoes et al., 2012) |
| 2016 | Mohammad Reza Haghshenas | Meta Analysis (Iran) | 1539 | 23.6% (6.7- 40.5) | PCR(GP5/GP6,L1,E6), | (Haghshenas et al., 2016) |
Breast cancer is responsible for death in women in all over the world and the incidence of mortality has increased in many Asian countries (Fard, 2012). In around the world, it has been estimated that more than a million women each year suffer to Breast cancer and more than 400,000 people die owing to it (Fernandes et al., 2015). The known risk factors for breast cancer are most commonly estrogen and growth hormones in women, including premature, late menopause, postmenopausal, obesity, and hormone therapy (Kolahdoozan et al., 2010; Yahyapour et al., 2013). Viruses are also the main suspects as etiologic agents of human breast cancer. MMTV; HPV and EBV are the first candidate viruses to be breast cancer (Aguayo et al., 2011; Glenn et al., 2012; Malekpour Afshar et al., 2015). The first evidence that HPV may be involved in breast cancer was shown by Di Lonardo et al in 1992, which has confirmed HPV type 16 in 29.4% of breast cancer samples (Dilonardo et al., 1992). The frequency of HPV in breast cancer is consistent in previous publications before 2009, reporting was shown the presence of HPV in breast cancer tissues of the world-wide have a prevalence ranging from 4–86% (Damin et al., 2002; Balekouzou et al., 2016). While HPV has been found in breast cancer tissues of women in many countries, the results are sometimes incompatible and methodology of the HPV testing has been questioned in some cases (Mendizabal-Ruiz et al., 2009; Mou et al., 2011; Herrera-Romano et al., 2012). In a U.S. study HPV was found in only three of 67 breast cancer cases and a French study no HPV was found in 50 breast cancer tissue samples, suggesting that HPV does not play an important role in frequently breast cancer (Gannon et al., 2015). There was some evidences for producing of breast cancer, Women with HPV-positive breast cancer have been reported to be significantly younger than women with HPV-negative breast cancer, suggesting that is a different etiology for involving HPV in younger women, High-risk HPVs generate two oncoproteins E6 and E7 which interfere with the cell cycle that afford formation stimulating colony and migration of breast cancer cells, also capable of the converting non-invasive breast cancer cells to invasive breast cancer cells, finally, the p53 tumor suppressor protein often is mutated in cancers, but not in tumors associated with HPV (Aceto et al., 2010). The high-risk HPV types, is associated with an increased risk of breast cancer, and this relationship is dramatically different among geographical areas, for example: the frequency of high-risk HPVs [types 16,
in breast cancer were, 46% in Norway, 15% in Europe, 35% in China, 6.5% in Korea, 14% in Brazil and in Australian 48% (Atique et al., 2017). In the present study was done a Real time PCR based on probe to aimed for detection of the HPV DNA in breast cancer tissues and non-cancerous tissues and INNo-Lippa was performed for HPV Typing based on inverse hybridization probe assay. Then, the association of human papillomavirus infection with clinical aspects in 98 cases of breast cancer has been analyzed. In this study out of 98 samples with non-cancerous tissues (Healthy group) all of them were negative for HPV. Similar other studies the presence of human papillomavirus in breast cancer tissues were confirmed. Doosti and colleagues were shown that Out of 87 tissue samples of breast cancer, 22.9% had human papillomavirus genome and no HPV DNA was found in 84 non-cancerous breast tissues, They used PCR and Nested PCR tests with specific primers to detect HPV (Doosti et al., 2016). In 2012, Sigaroodi and colleagues conducted a study in northern Iran, in this study; 130 Paraffin block (79 samples with breast cancer and 51 non-cancerous as “control”) were examined by PCR method, from a total of 79 samples of breast cancer, 15 cases (25.9%) was positive that of these 53.34% were positive for HPV type 16 and 18 and the other HPV genotypes were 6, 11, 15, 23. Out of the 51 non-cancerous samples, only one sample (2.4%) was positive for HPV type 11 (Sigaroodi et al., 2012).

In high-risk group, HPV Types 16 and 18 were and in low-risk group HPV types 6 and 11 were the most frequent.

In conclusion, although researchers have connected HPV to cervical cancer, suggesting a link exists between breast cancer and HPV is controversial, it’s difficult to determine a link between HPV and breast cancer and, further studies involving a larger number of samples and the evaluation of other parameters are necessary. In this study, HPV genome was found in 8.2% of all breast cancer specimens, there was no significant difference between HPV and age, but there was a significant relation between HPV, type of HPV and grade of tumor. Further studies are needed to clarify the role of HPV in breast cancer in Iran, So, if HPV was a risk factor for breast cancer, solutions such as antiviral therapy or vaccination prevention can be effective.

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