RESEARCH ARTICLE

No Detection of 'High-risk' Human Papillomaviruses in a Group of Iranian Women with Breast Cancer

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Abstract

The presence of viral DNA in breast cancer cells is controversial. However, some studies have revealed a possible role for the human papillomavirus in the pathogenesis of breast cancer. The aim of the present study was to investigate the presence of HPV-DNA in breast tissue in a group of Iranian women with and without breast cancer and identification of the detected HPV types. Paraffin-embedded specimens from 65 malignant breast cancer cases and 65 cases with benign breast lesions were investigated for presence of HPV-DNA by nested polymerase chain reaction. We found HPV-DNA in 22 (33.8%) of the breast cancer specimens. All non-cancerous specimens were negative. Low and high-risk HPV types, including HPV-6 (26.2%), HPV-16 (1.5%), HPV-35 (1.5%), HPV-52 (1.5%), and HPV-11 (1.5%) were detected in our study. HPV-6 was the most prevalent type in the breast cancer specimens. Although high-risk HPV types have been shown to have a major role in cervix cancer, there have been no data that support the same relevance for other types of malignancies. Furthermore, presence of low-risk HPV types in malignancies still is a matter of debate. The data presented in this study indicates a strong need for epidemiological studies correlating different HPV types in human breast cancer.

Keywords: Human papillomavirus - breast cancer - Iran

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Introduction

Breast cancer is the second most common cancer in the world, accounts for 25% of all cancers (excluding non-melanoma skin cancers) in women (GLOBOCAN, 2012). According to recently published data, the overall cancer incidence in Iran has been 110 per 100,000 in men and 98 per 100,000 in women in 2006. Among Iranian women, breast cancer is the leading cause of cancer morbidity and mortality, even though its incidence is among the lowest in the world, i.e., 25 per 100,000 (Mousavi et al., 2008).

Breast cancer affects Iranian women at least one decade younger than their counterparts in developed countries (Harirchi et al., 2004). There are many published studies about breast cancer in Iran, but the epidemiological aspects of Iranian breast cancer are uncertain.

Well known risk factors of breast cancer are genetic and epigenetic factors including: mutations in breast cancer susceptibility genes BRCA1 and BRCA2, TP53, sex-steroid hormones and lifestyle factors (Hulka and Moorman, 2008; Derks-Smeets et al., 2014). Other potential risk factors like lack of childbearing or breastfeeding (Joshi et al., 2012), environmental agents, histology of benign lesions, ionizing radiation, mammographic density and other factors (Dumitrescu et al., 2005; Hulka and Moorman, 2008) have been strongly implicated in the development of breast cancer. The mechanism(s) of breast carcinogenesis is still not clearly understood (Hedau et al., 2011).

The search for a viral cause for human breast cancer has generated considerable controversy (Lawson et al., 2006; Hachana et al., 2010). Bittner's discovery in 1942 that mouse mammary tumor virus (MMTV) caused mammary cancers in mice (Bittner, 1942) inspired scientists to postulate that a virus might also cause human breast cancer. Three viruses that have been most studied as possible causes of human breast cancer are mouse mammary tumor virus-like sequences (MMTV-LS), Epstein-Barr virus (EBV), and oncogenic (high-risk) types of human papillomavirus (HPV) (Joshi and Buehring, 2012).

The association between human papillomavirus (HPV)

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and anogenital tumors, especially cervical cancer, is well documented. The relationship between genital HPV types and breast cancer is unclear; however, it has been shown that HPV types 16 and 18 can immortalize normal breast epithelium (Wrede et al., 1992).

Di Lonardo et al. (1992) were the first to report the relationship between HPV and breast cancer, demonstrating HPV-16 DNA in 29.4% of 17 samples of breast carcinoma analyzed by PCR. Hennig et al. (1999) studied women with both a history of high grade cervical intraepithelial neoplasia (CIN III) and breast carcinoma as a second primary neoplasia also using PCR. They detected HPV-16 DNA in 19 of 41 cases (46%) of breast cancer, suggesting that HPV associated cervical neoplasia might be the original site of HPV infection from where the virus could be transported to the breast. Additionally, HPV-18, -31 and -33 were also detected in breast cancer specimens (Yu et al., 2000). In contrast, other authors failed to demonstrate the presence of HPV DNA in samples of breast carcinoma, even using the same methods of detection (Gopalkrishna et al., 1996; Hachana et al., 2010). Considering these controversial data and following our previous study (Shahmahmoudi et al., 2007), our aim was to investigate the presence of HPV–DNA of high and low-risk mucosal types in female breast carcinomas as well as in non-cancerous breast tissues of a group of Iranian women by nested PCR.

Materials and Methods

Patients and tissue samples

Formalin-fixed paraffin-embedded tissues from 65 malignant breast cases and 65 cases with benign breast lesions were investigated by Nested-PCR.

All samples were obtained from Department of Pathology in the city of Tabriz, located in the East Azerbaijan in Iran. Tissue samples were cut by separate scalpels and collected in sterile tubes. To avoid contamination between various specimens, special care was taken to change gloves and scalpel before cutting each block.

DNA extraction and HPV detection

Deparaffiniation was performed by rotation overnight in 1 ml of xylene, followed by centrifugation and subsequent removal of the supernatant. This step was repeated twice (each for one hour) with fresh Xylene. Xylene was in turn removed twice by rotation in 1 ml of 100% ethanol for five minutes at 37°C, followed by centrifugation and subsequent removal of the supernatant. Pellet was dried at 37°C. Thereafter, the tissue was lysed in digestion buffer, consisting of 200μg/ml proteinase K, then incubated at 56°C for 1 hour, and the incubation was continued at 37°C until samples were fully dissolved, which took usually 24-30h. Then all samples were processed with "QIAamp DNA FFPE tissue kit" according to the manufacturer's instructions (Qiagen, Hilden, Germany). For each sample β-globin gene primers were used as internal control to assess the quality of DNA. Primers for HPV L1 genome region were used for HPV detection by nested–PCR (de Roda et al., 1995; Othman and Othman, 2014) (Table 1). DNA sample of a patient who was known to be positive for HPV-11 and sterile distilled water were used as positive and negative controls, respectively. Nested-PCR was performed according to protocols mentioned in previous studies (Shahmahmoudi et al., 2007).

The amplified products were visualized on 2.5% agarose gel. PCR was repeated for each HPV positive sample to make sure of the result.

Sequencing

In order to confirm the results and detection the HPV type, PCR products were submitted to direct sequencing. PCR products were purified with prep PCR Purification Kit (Bioneer, Korea). After purification, products were sequenced in a 3130 Genetic analyzer ABI. HPV type was determined using NCBI- BLAST.

Results

Tissue sections taken from paraffin-embedded blocks from 65 breast cancer cases and 65 non-cancerous breast specimens were available for analysis by PCR for the presence of HPV DNA. DNA extracted from tissue sections was positive for β-globin gene in all specimens, indicating that quality and quantity of DNA was satisfactory.

The characteristics of study subjects including age and histopathologic types in case and control groups are shown in Table 2. 63.1% and 74.6% of specimens in case and control groups belonged to women less than 50 years of age. The most frequent type of breast cancer determined

Table 1. Primer Sequence and Size of the Amplicon

Primers	Primer sequence	Location	Amplicon size
MY09	5'-GCM CAG GGW CAT AAY AAT GG-3'	L1	450 bp
MY11	5'-CGT CCM ARR GGA WAC TGA TC-3'		
GP5+	5'-GTT ACT GTG GTA GAT ACT AC-3'	L1	150 bp
GP6+	5'- CTT ATA CTA AAT GTC AAA TAA AAA-3'		
PCO4	5'-AACTTCATCCACGTTCACC-3'	Beta Globin	250 bp
GH20	5'- GAAGAGCCAAGGACAGGTAC-3'		

Table 2. Age Group and Histopathological Characteristics in Breast Cancer Cases and Control Groups (n=65)

		Cases %	Control %
Age group	<40	20 (30.8%)	39 (45.4%)
(years)	40-50	21 (32.3%)	19 (29.2%)
	>50	24 (36.9%)	7 (10.8%)
Histopathological characteristics	IDCIa	6 (9.2%)	-
	IDCII	36 (55.4%)	-
	IDCIII	8 (12.3%)	-
	ILCI b	3 (4.6%)	-
	ILCII	8 (12.3%)	-
Phyllodes tumor		1 (1.5%)	-
Metastatic tumor		1 (1.5%)	-
Mucinous carcinoma		1 (1.5%)	-
Fibrocystic		-	53 (81.5%)
Fibroadenoma		-	6 (9.2%)
Lipoma		-	1 (1.5%)
Fat necrosis		-	1 (1.5%)
Epidermal cystic		-	2 (3.1%)
Lymph node		-	1 (1.5%)
Ruptured epidermal cystic	-	1	(1.5%)

Table 3. Frequency of HPV Types and Histopathological Characteristics in Cases with Breast Cancer

Histopathological characteristics	HPV-6 No. (%)	HPV-11 No. (%)	HPV-16 No. (%)	HPV-35 No. (%)	HPV-52 No. (%)	Negative No. (%)
IDCI[a]	2 (11.9%)	-	-	-	-	4 (9.1%)
IDCII	12 (70.4%)	-	-	-	-	24 (54.5%)
IDCIII	1 (5.9%)	-	1 (100%)	-	1 (100%)	5 (11.4%)
ILCI[b]	1 (5.9%)	-	-	-	-	2 (4.5%)
ILCII	1 (5.9%)	-	-	-	-	6 (13.6%)
Phyllodes tumor	-	-	-	1 (100%)	-	1 (2.3%)
Metastatic tumor	-	-	-	-	-	0 (0.0%)
Ductal carcinoma	-	1 (100%)	-	-	-	1 (2.3%)
Mucinous carcinoma	-	-	-	-	-	1 (2.3%)
Total (100%)	17	1	1	1	1	44

histologically was Invasive Ductal Carcinoma II (IDC II) (55.4%). In control group, the most frequent lesion was Fibrocystic (81.5%) (Table 2).

Twenty- two out of 65 (33.8%) breast cancer biopsies were positive for HPV-DNA. In non- cancerous specimens no HPV-DNA was detected. Sequencing of positive specimens showed them to be variants of HPV-6, -11, -16, -35 and -52 (Table 3). Sequencing of one positive sample failed. HPV type and breast cancer histopathologic types are shown in Table 3. The most frequent HPV type was HPV-6 which was mainly found in IDC II.

Discussion

After lung cancer, breast cancer is the most common cancer in the world, affecting one in eight women during their lifetime (Parkin, 2005). The role of viruses in the etiology of breast cancer has been an area of interest for over years.

It is well accepted that in addition to cervical cancer, some of different human organs, including breast, harbor HPV-DNA (Damin et al., 2004). However, there are studies evaluating the controversial presence of Human papillomavirus in breast lesions and the reported prevalence of HPV infection in breast cancer shows a great variation worldwide. Demographic features, genetic backgrounds, numbers of samples tested, methodological differences, low viral loads and the sensitivity of methods used, may be attributed to the difference of HPV prevalence in breast carcinomas in different reports (Khan et al., 2008). Still, there is insufficient data about the role of HPV in breast cancer.

Following our previous study on detection of mucosal HPVs in non-melanoma skin cancer (Shahmahmoudi et al., 2007), the aim of the present survey was to investigate the presence of HPV-DNA and determination of HPV type in breast tissues in a group of Iranian women with and without breast cancer. Paraffin-embedded specimens from 65 malignant breast cancer cases and 65 cases with benign breast lesions were investigated for presence of HPV-DNA by nested-polymerase chain reaction. HPV-DNA was found in 22 (33.8%) breast cancer specimens. Low and High-risk HPV types, such as HPV-6, -11, -16, -35 and -52 were detected in our study and HPV-6 was the most prevalent type, mainly found in Invasive Ductal Carcinoma.

The means by which HPVs are transmitted to the breast are not known. Human papilloma virions are known to be released when the cornified envelope of cells desquamate; accordingly, HPVs can be transmitted by skin-to-skin contact as well as by sexual activity (Bryan and Brown, 2001). Lawson et al. (2009) reported that cell surface-to-surface contact mainly during sexual activities is required for HPV transmission and modulation of cellular pathways. According to a study, HPV is mainly found on the dermis of the breast and travels through the nipple and ducts, infects the breast tissue and starts the potential malignant process (De Villiers et al., 2005). Since the mechanism is not clear, it is quite probable that additional cofactors are needed to immortalize and transform the infected breast cells (Lindel, 2007).

In our study none of the non-cancerous specimens were positive for HPV-DNA. Another study conducted in north part of Iran detected HPV-DNA in 25.9% of breast cancer and 2.4% of women with non-cancerous status with HPV-16 and -18 were the dominant types in cancerous specimens (Sigaroodi et al., 2012). Earlier studies on breast cancer have reported HPV types-11, -16 and -18 are the most frequent in women living in the United States and Brazil (Damin et al., 2004), and HPV-18 is present in the majority of Australian women (Lindel et al., 2007). In parallel, HPV-33 is the most frequent virus in Asian women (Yu et al., 2000). HPV-16 has been identified in Italian and Norwegian women who had previous cervical neoplasia (Hennig et al., 1999). Studies on the presence of high-risk HPV types in the Middle East reveal that HPV-18, -33 and -35 are present in breast cancer and normal mammary tissues in Turkish women (Shucla et al., 2009). In our study we detected HPV-6 in 17 (26.2%) cases with breast cancer and the only high-risk type was HPV-16 which was detected in only 1 case (1.5%). This data is approximately in accordance with the reports that found no evidence of high-risk HPVs in breast cancer (Gopalkrishna et al., 1996; Hachana et al., 2010; Hedau et al., 2011).

Methodological differences play an important role in detection of HPV DNA in breast carcinomas. The best way for the laboratory diagnosis of HPV are molecular tests based on detection of viral DNA in the tissue. Concentration of HPV in breast tumors seem to be much lower than its concentration in cervical cancer, which makes the detection of HPV sequences in breast tumors more difficult (Gumus et al., 2006). Therefore, using a highly sensitive method for HPV-DNA detection in breast tissues is crucial. It has been shown that nested PCR using MY09/11 and GP5+/6+ as outer and inner primers, respectively, is much more sensitive than solely MY or GP primers in detecting mucosal HPV types (Husnjak et al., 2000). In our previous study (Shahmahmoudi et al., 2007), we detected mucosal types of HPV in skin lesions by using the above mentioned primers; so we chose to use the same nested PCR protocol for breast tissues as well.

HPV-6 and HPV-11, which were detected in malignant breast tissues in our study, are regarded as low-risk HPV types because they have only rarely been demonstrated in premalignant or malignant tissues (Panigoro et al., 2013; Sui et al., 2013). However, introduction of HPV-6 E6 into normal mammary cells leads to immortalization and reduced levels of the P53 protein (Kitasato et al., 1994).

The molecular mechanism by which these low-risk HPV types induce or participate in the transformation of cells has not been resolved, although there are theories on this issue (Doorbar et al., 2012).

The modest sample size of the study groups was the main limitation of present study. A future study including a large population from different regions of Iran is desirable in order to determine the frequency of HPV in Iranian women with breast cancer.

The data presented in all studies indicate a strong need for more epidemiological studies correlating different HPV types in human breast cancer. The mere presence of the low-risk HPV-6 in breast carcinoma samples in this study and other reports of low and high-risk HPV types in breast tissues do not prove their role in the etiology of the disease; yet, it demands for further investigation.

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