Introduction

Breast cancer is the leading type of cancer in women worldwide. Roles of the Epstein-Barr, Merkel cell polyoma and mouse mammary tumor viruses in breast carcinogenesis are still controversial although any relationship would clearly be important for breast cancer etiology, early detection and prevention. In the present study associations between EBV, MMTV and Merkel cell polyoma virus and breast cancer in 100 Iranian patients were evaluated using paraffin-embedded tissues. EBER RNA and expression of p53 and large T antigen were evaluated by real time PCR and CD34, p63, HER2, PR and ER markers were studied by immunohistochemistry. EBV was detected in 8/100 (8%), MMTV in 12/100 (12%), MPy in 3/100 (3%) and EBER RNA in 18/100 (18%) cases. None of the control samples demonstrated any of the viruses. p53 was suppressed in EBV, MPy and MMTV positive samples. The large T antigen rate was raised in MPy positive samples. Our results showed that EBV, MMTV and the Merkel cell polyoma virus are found with some proportion of breast cancers in our patients, suggesting that these viruses might have a significant role in breast cancer in Kerman, southeast of Iran.

Keywords: Breast cancer - Epstein-Barr virus - Merkel cell polyoma virus - mouse mammary tumor virus

Evaluation Frequency of Merkel Cell Polyoma, Epstein-Barr and Mouse Mammary Tumor Viruses in Patients with Breast Cancer in Kerman, Southeast of Iran

Malekpour Afshar Reza1, Mollaie Hamid Reza2*, Lashkarizadeh Mahdiyeh3, Fazlalipour Mehdi4, Zeinali Nejad Hamid5

Abstract

Breast cancer is the most common cancer among women worldwide. Roles of the Epstein-Barr, Merkel cell polyoma and mouse mammary tumor viruses in breast carcinogenesis are still controversial although any relationship would clearly be important for breast cancer etiology, early detection and prevention. In the present study associations between EBV, MMTV and Merkel cell polyoma virus and breast cancer in 100 Iranian patients were evaluated using paraffin-embedded tissues. EBER RNA and expression of p53 and large T antigen were evaluated by real time PCR and CD34, p63, HER2, PR and ER markers were studied by immunohistochemistry. EBV was detected in 8/100 (8%), MMTV in 12/100 (12%), MPy in 3/100 (3%) and EBER RNA in 18/100 (18%) cases. None of the control samples demonstrated any of the viruses. p53 was suppressed in EBV, MPy and MMTV positive samples. The large T antigen rate was raised in MPy positive samples. Our results showed that EBV, MMTV and the Merkel cell polyoma virus are found with some proportion of breast cancers in our patients, suggesting that these viruses might have a significant role in breast cancer in Kerman, southeast of Iran.

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RESEARCH ARTICLE
Immunohistological stain

We performed Immunohistochemistry analyses on 100 cases of invasive ductal carcinoma. Patients included in this study were from hospitals in the city of Kerman (Afzalipour, Shahid Bahonar) and private laboratories. Formalin-fixed, paraffin blocks of tumor and non-tumor tissue sections from each patient were re-examined by pathologists and classified according to the Nottingham combined system histological grading scheme which included mitotic rate, nuclear and structural properties. Specimens were classified as grades 1, 2, and 3. The block from each tumor sample with areas of invasive cancer, DCIS, atypical ductal hyperplasia and flat epithelial atypia, (DIN Ia, Ib), fibrocystic changes and normal areas were selected for further analyses. If these were not located on one block, the specific areas of the different blocks were cut and placed on one slide. Serial sections of 5 μm were obtained from each block and mounted on silanized slides for immunohistochemical staining. Dehydrated, deparaffinized sections along with retrieval buffer were microwaved for 20 min (3 min at 850 watts; 17 min at 180 watts), then blocked for 10 min with 0.5 % H2O2. Sections were incubated for 1 hour at room temperature with the monoclonal antibodies as follows: ER (1:50; DAKO, Clone 1D5); PR (1:100; DAKO, Clone PgR 636); HER2-neu (1:100; DAKO), CD34 (1:100; DAKO, Clone QBEnd 10); CD34 (1:100; Novocastra, Clone QBEnd 10); and SMA (DAKO, Clone 1A4): Ready to use. Slides were rinsed with wash buffer for 5 min, which was repeated twice, between all main stages. Envision polymer (30 min) was added using 3,3’-diaminobenzidine (DAB) as the chromogen (10 min) followed by staining with hematoxylin for 2 min, dehyradation and mounting the slides.

Deparaffination samples

Paraffinized blocks from the 100 tumor samples and normal tissues were cut in 5-μm sections and 8 sections, patients were collected in the same micro-centrifuge tube. Samples were de-waxed in 500 μl xylene; All micro-centrifuge tube located about 10 min in a 60 °C heated block and centrifuged at 8,000 RPM, the supernatant was removed. This step was then repeated 3 times. Add 500 μl absolute ethanol, centrifuge at 10,000 RPM for 1 min, the samples were then dried in a 60°C heated block with open lids for 10-20 min for remove residual ethanol.

Tissue digestion

According to samples (biopsy or Paraffinized blocks), 200-400 μl of Tissue Lysis Buffer was added to each tube [4 M Urea, 200 metros, 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 and RNA 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 QIAamp DNA Mini kit (Qiagen, Germany) and Total RNA were
Frequencies of MCV, EBV and MMTV Positivity in Breast Cancer in Kermanshah, Iran

Virus detection uses real time PCR

Real time PCR was carried out using EBV, MPy and MMTV Real Time PCR kit (Inter Lab Service, Russia) following instruction manual.

rReal Time PCR

For the determination, the expression level of EBER, p53 and large T-antigen mRNA, reverse transcription real time PCR (rReal Time PCR) was carried out by using the first strand cDNA synthesis kit by Revert Aid cDNA synthesis kit (Thermoscientific, USA). Briefly, RNA samples were heated to 65°C for 10 minutes and then chilled on ice. The uniform suspension of bulk first-strand cDNA reaction mix was added according to the manufacturer’s protocol.

One μl of DTT solution, and 1 μl of random Hexamer (N) 6 primer (0.2 μg) were then added to the heat-denatured RNA. Samples were mixed properly by pipetting up and down several times and then incubated for 1 hour at 42°C. For Real time PCR, the QuantiTect Probe PCR Kit (Qiagen, Germany) used base on instruction kit. Real time PCR primers and probes were designed for EBER, p53 and T antigen mRNA after alignment of these regions between all of them in EBML-EBI and as an internal control, β-Actin were purchased from Metabion company (Germany) (Table 1).

Statistical analysis

Chi-square test or Fisher’s exact test was conducted using SPSS version 17 for the association between the presence of DNA virus genome and other characterizes (values P=0.05 were considered statistically significant).

Results

In our study, 100 female with breast cancer (Invasive, DCIS, DIN) and 100 breasts normal tissues (mean age 49.03±11.58) were used. We included 100 females diagnosed with the invasive ductal carcinoma of the breast; with a mean age of 49.1 ± 1.1 years and mean tumor size of 3.2 ± 0.2 cm. The mean number of the involved axillary lymph nodes was 8.1 ± 0.8 and the following tumor grades were reported: grade I (35%), grade II (42%), and grade III (23%) (Table 2). The Real-time PCR results were shown in Table 3 and as it is clear, 8 cases (8%) were EBV DNA positive in breast cancer samples and 10 cases (22.2%) was positive in lymph nodes. EBER RNA expression rate was positive in 18 (%) cases in breast cancer samples and 25 (55.5%) cases in lymph nodes. For MMTV DNA, 12 (12%) was positive in breast cancer samples and 14 (71.4%) was positive in lymph nodes. Positive Real-time PCR result in MPy DNA was 3 (3%) in breast cancer samples and 8 (17.7%) in lymph nodes. The expression of p53 was shown in Figure 1 and as it is clear, the rate of p53 was down regulated in EBV, MPy and MMTV positive samples comparing to negative samples.

Large T antigen expression level was evaluated in Figure 2 and sharp raised level was detected in MPy positive samples (0.8), although slight rate of LTag was detected in other samples. In Figure 3, the expression rate of EBER RNA was shown and based on our expectation it was high in EBV positive samples and very slight low level was detected in MPy and EBV negative samples. No expression was shown in negative MPy and MMTV positive and negative samples. Based on Figure 4, different Immunochemistry markers were evaluated in invasive tumors and the rate of CD34 was shown on top that shows the increased level and after that the rate of

Table 1. Sequence Primer and Probes for Real Time PCR in this Study

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53</td>
<td>CAGCATTTATCCGAGTG</td>
<td>GATGGTGTTACAGTCAGA</td>
<td>CCAACCTCAGCCGGCTCATA</td>
<td>147-269</td>
</tr>
<tr>
<td>Large T antigen</td>
<td>GCAGCCATATGTTGCTGATG</td>
<td>CCTGAGATGCTCTTGCT</td>
<td>ACTCTAACCCTCTCTCTCATA GAGCA</td>
<td>201-272</td>
</tr>
<tr>
<td>EBER</td>
<td>TGAGGAGATGATACCTGTGA</td>
<td>CTGTACTTGGACCCGAGA</td>
<td>ACCCTCAGGACCTAGCTG</td>
<td>138-321</td>
</tr>
</tbody>
</table>
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*p63 was detected. Other markers such as ER, PR and HER2 were detected at low level. In Figure 5 different Immunochemistry markers were evaluated in DCIS Tumors and in first position the highest level of CD34 detected and the p63 was shown in second position. Other markers such as ER, PR and HER2 were detected at low level. Different Immunochemistry markers were evaluated in DIN Tumors in Figure 6 and highest level of*  

**Table 2. Patient and Tumor Characteristics**

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>No (%)</th>
<th>Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50</td>
<td>60 (60)</td>
<td>49.1±1.1</td>
</tr>
<tr>
<td>&gt;50</td>
<td>40 (40)</td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>No (%)</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>&lt;2</td>
<td>17 (17)</td>
<td>3.2±0.2</td>
</tr>
<tr>
<td>5-Feb</td>
<td>70 (70)</td>
<td></td>
</tr>
<tr>
<td>&gt;5</td>
<td>13 (13)</td>
<td></td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>No (%)</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>&lt;3</td>
<td>13 (28.9)</td>
<td>8.1±0.8</td>
</tr>
<tr>
<td>&gt;3</td>
<td>32 (71.1)</td>
<td></td>
</tr>
<tr>
<td>Tumor grade</td>
<td>No (%)</td>
<td>StDev</td>
</tr>
<tr>
<td>1</td>
<td>35 (35)</td>
<td>0.327</td>
</tr>
<tr>
<td>2</td>
<td>42 (42)</td>
<td>0.344</td>
</tr>
<tr>
<td>3</td>
<td>23 (23)</td>
<td>0.438</td>
</tr>
</tbody>
</table>

**Table 3. Real timePCR results for EBV, MMTV, MPy and EBER**

<table>
<thead>
<tr>
<th>Test</th>
<th>Results in Breast cancer</th>
<th>Results in Normal tissue</th>
<th>Results in Lymph nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pos (%)</td>
<td>Neg (%)</td>
<td>Pos (%)</td>
</tr>
<tr>
<td>EBV DNA</td>
<td>8 (8)</td>
<td>92 (92)</td>
<td>0(0)</td>
</tr>
<tr>
<td>MMTV DNA</td>
<td>12 (12)</td>
<td>88 (88)</td>
<td>0(0)</td>
</tr>
<tr>
<td>MPy DNA</td>
<td>3 (3)</td>
<td>97(97)</td>
<td>0(0)</td>
</tr>
<tr>
<td>EBER RNA</td>
<td>18 (18)</td>
<td>82(82)</td>
<td>0(0)</td>
</tr>
</tbody>
</table>

![Figure 3. Relative Expression of EBER RNA in Different Samples](image)

**Figure 4. Distribution of Immunochemistry markers in Invasive Tumors**

**Figure 5. Distribution of Immunochemistry markers in DCIS Tumors**

**Figure 6. Distribution of Immunochemistry markers in DIN Tumors**
CD34 detected in high position and after that the p63 was shown in second position. Other markers such as ER, PR and HER2 were detected at low level.

**Discussion**

Breast cancer is a leading cause of death in women worldwide and has been indicated that the incidence of this malignancy is rising approximately 3% per year (Forouzanfar et al., 2011). Environmental factors have an important role in the etiology of breast cancer. From the environmental factors, viruses have received considerable attention and also a number of viruses have been implicated in the pathogenesis of breast cancer, including mouse mammary tumour virus (Fernandez et al., 2007; Darbre and Fernandez, 2013) merkel cell polyomavirus and Epstein- Barr virus (Paredes et al., 2005; Mazouni et al., 2011). However, it is not clear that the virus has a central role in the pathogenesis of this malignancy.

The association of EBV, MMTV and Merkel cell polyomavirus with breast cancer comes from data which shows that: (a) they are present in breast tissue, detected in breast milk; (b) their DNA stimulates growth of human breast milk cells; (c) some viral-associated lymphomas occur in the breast. EBV has a high prevalence of breast carcinomas and, in endemic areas, positive samples showed higher loads of the EBV genome (Oskouee et al., 2014; Yahia et al., 2014; Mazouni et al., 2015). The role of different viruses causing human BC has been investigated with variable results, providing evidence in favor or against it, hence the issue remains controversial. Many researchers have detected the EBV, MMTV and Merkel cell polyomavirus by using real time PCR in human breast cancers (Wang et al., 2014).

But others did not detect the MMTV in 18 human breast cancers they examined Bindra and colleagues found only 1 of 60 human breast cancers by PCR (Bindra et al., 2007). Witt and colleagues tested 50 Austrian human breast cancer samples and did not detect the MMTV env sequence in any of them (Witt et al., 2003). Mant and colleagues detected 16% of 44 human breast cancer samples, but all were false positives (Mant and Cason, 2004). In 2008, evaluated the presence of MMTV- like virus in invasive breast cancer in 46 Japanese women by PCR and Southern blot hybridization and did not detect any virus particle in the tissue specimen tested (Fukuoka et al., 2008). However, Ford CE et al detected very low ratio of MMTV DNA in Vietnamese and Vietnamese-Australian women (0.8% and 0%, respectively) using PCR method (Ford et al., 2004). Molecular method of the virus detection may also change the rate of virus detection in samples. Presence of MMTV-like virus env gene sequence in Italian women by fluorescence nested PCR method which was positive in 33% of human breast cancer cases(Melana et al., 2002). Formalin used for preparation of formalin-fixed paraffin-embedded tissues may fragments the DNA and interfere with PCR amplification of large sequence of DNA templates. A simple way for reducing the effect of DNA degradation on the detection in specimens is targeting of short DNA sequences (<200 base pair) in PCR. In our study, Real Time PCR for amplification has been used as a highly sensitive and specific method for evaluation (Baltzell et al., 2012).

We have detected EBV, MMTV and Merkel cell polyomaviruses positivity in 100 breast cancer tissues and the rate were (EBV 8%), (MMTV 12%) and (MPy 3%) in Iranian patients and also it has been shown that from 100 lymph node tissues, EBV (22.2%), MMTV (71.4%) and Merkel cell polyomavirus (17.7%) were positive by real time PCR. This data indicates that EBV, MMTV and Merkel cell polyomaviruses have an important role in breast cancer in Iran. Earlier reports confirm that expression of EBERs can promote the tumorigenic phenotype in infected cell and in our study the rate of EBER RNA was 18% in breast cancer samples and 55.5% in lymph nodes samples which confirmed the role of EBER RNA in cell transformation (Naderimagham et al., 2014; Veisy et al., 2015).

Our data proposed that EBV, MMTV and Merkel cell polyomavirus DNA presence in breast cancer patients can differ between groups with different ages and population characteristics and the size of tumors and ages can be varied in population. The prevalence of EBV, MMTV and Merkel cell polyomavirus nucleotide sequence in Iranian breast cancer patients could be detectable. In 2012, studied MMTV-like sequences in Iranian breast cancer in Shiraz, in southwest Iran and found no virus (Motamedifar et al., 2012). The p53 gene has cell cycle controlling role and p53 suppression by viral activity is leading to the development of neoplasm. Based on this fact we could show that the p53 rate in positive EBV, MMTV and Merkel cell polyomavirus cases were dropped comparing to negative samples (Vernet-Tomas et al., 2015). The oncogenic potential of MCPy Large T antigen with the cellular retinoblastoma tumor suppressor protein (pRb) has been proposed and in our study the increased expression rate of LTag in positive MCPy cases detected comparing to other positive and negative samples (Goetz et al., 2001). In a study, the env sequences inpre invasive lesions for the presence of MMTV was found in 19% of normal epithelial cells collateral to ductal carcinomas in situ (DCISs) or infiltrating ductal carcinomas (IDCs), 27% of atypical ductal hyperplasias, 82% of DCISs, and 35% of IDCs (Mazzanti et al., 2011). In another study, founded MMTV in 44 (72%) of 61 paraffin-embedded IBC tumors, but did not compare with non-IBC tumors (Holland and Pogo, 2004).

Distribution of Immunochemistry markers such as CD34, p63, HER2, PR and ER were evaluated in Invasive Tumors, DCIS Tumors, DIN Tumors and Normal Tissues (Dabiri et al., 2013). In our study, CD34 and p63 have varied range compared to other markers. The mean rate of CD34 marker dropped in Invasive Tumors, DCIS Tumors, and DIN Tumors compared to normal cells, but on the other hand the increased level of p63 was shown in Invasive Tumors, DCIS Tumors, and DIN Tumors compared to normal cells. Further research is necessary to determine the role of EBV, MMTV and Merkel cell polyomavirus in the progression of breast cancer. These viruses were detected in the lymphocytes around the breast tissues, suggesting the contribution as a modulating agent in the microenvironment area around those breast tissues. Carcinogenesis can involve several factors and the role of...
viruses in most common cancers is certainly rare. Viruses can act as direct transforming agents or cofactors. The most probable mechanism of carcinogenesis may involve a combination of genetic alterations, immune system dysfunctions, and viral infections. It is recommended that finding the exact relationship between EBV, MMTV and Merkel cell polyomavirus and breast cancer in Iranian patients needs further studies with an increased number of patients.

In conclusion, our findings suggest that EBV, MMTV and Merkel cell polyomavirus might have a role in the pathogenesis of breast cancer. These findings were based on EBV, MMTV and Merkel cell polyomavirus detection by real-time PCR technique in breast cancer patients. So EBV, MMTV and Merkel cell polyomavirus may contribute to increased tumor aggression in patients from different ages and different geographic region.

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References


