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Detection of Epstein Barr Virus among Benign and Malignant Breast Tumors

[1]M. Eghbali, [1]M. Ghane, [1]M. Mirinargesi and [2]A. Zare Mehrjardi
[1]Department of Microbiology, Tonekabon Branch, Islamic Azad University, Tonekabon, Mazandaran, Iran
[2]Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

Corresponding Author: M. Eghbali, Department of Microbiology, Tonekabon Branch, Islamic Azad University, Tonekabon, Mazandaran, Iran Tel: 0098 9111936373

ABSTRACT

Epstein Barr Virus (EBV) infects more than 90% of the world's human population. An association is proposed between EBV infection and occurrence of breast cancer, with a large difference in the results reported by different researchers. This study aimed to investigate the frequency of EBV among benign and malignant breast tumors. A total of 24 carcinomas and 24 fibroadenomas paraffin embedded tumoral tissue samples were obtained from the pathology sections of Toos and Firoozgar hospitals in Tehran, Iran. All samples had been collected from patients from June 2011 to February 2012. DNA was extracted from all samples and their infection with EBV was examined by PCR technique. The results obtained by this study showed that 4 out of 24 carcinoma samples (16.6%) were infected by EBV, while the number of fibroadenoma samples infected by this virus was 1 (4.1%). The frequency of EBV infection was different among malignant and benign tumors. However, no association was observed between EBV infection and the formation of malignant or benign tumors based on the Chi-square test. In relation to some other studies, this analysis does not confirm any association between EBV infection and breast cancer occurrence. However, due to the high frequency of EBV infection among breast cancer patients, future studies are needed to elucidate the possible role of the virus in the disease.

Key words: Breast cancer, carcinoma, fibroadenoma, Epstein Bar Virus, PCR

INTRODUCTION

Breast cancer is the uncontrolled growth of abnormal cells, which is created in various regions of the breast. This cancer may develop in different tissues such as ducts, which transfer breast milk, production tissue of the milk and in non-glandular tissue. Breast cancer is the most prevalent cancer in women (Parkin *et al*., 2005). Every year, a large number of women are afflicted by breast cancer, of which the disease is fatal for a number of sufferers (Khorshid, 2011). According to the data presented by the US National Institute of Cancer, out of every 8 women, one individual will be inflicted with breast cancer (Wong *et al*., 2002). Although genetic factors, such as mutation in the BRCA1 and BRCA2 genes, play an important role in the occurrence of the cancer (Miki *et al*., 1994; Wooster *et al*., 1995), other susceptible factors cannot be neglected. For example, the risk of suffering from breast cancer increases with age (Abbasi *et al*., 2009; Alamelumangai and Devishree, 2012). Almost, three quarters of the cases of affliction occur in women who are more than 50 years old. Except for age, other risk factors include: family history of breast cancer,

puberty before the age of 13 years, menopause after the age of 51 years, women who have never become pregnant and those who have become pregnant for the first time after the age of 30 years, suffering from obesity, particularly after menopause, poor diet (Hafidh *et al*., 2009), excess alcohol consumption and viral infection (Wooster *et al*., 1995).

The studies carried out in two recent decades have provided the role of viruses in the occurrence of breast cancer. One of the most important viruses is the Epstein Barr Virus, which is a member of the herpes viridea family. Attributes of this virus have a double strand DNA and icosahedral symmetry. This nucleocapsid has been surrounded by a covering being of the lipid genus type (Izham *et al*., 2011). EBV is a carcinogenic virus and known as one of the prevalent viruses around the world. At present, this virus is identified as the cause of a number of varieties of cancer, including African Burkitt's lymphoma (Epstein *et al*., 1964; Irshaid *et al*., 2010), Hodgkin lymphoma, nasopharyngeal carcinoma (Nikakhlagh *et al*., 2010), Lymphoproliferative disorder (Hsu and Glaser, 2000; Niedobitek *et al*., 2001) and gastric carcinomas (Perrigoue *et al*., 2005). The genetic material of the EBV was identified for the first time in the breast tumors of Japanese women by Horiuchi *et al*. (1994). After that, more than a hundred studies were conducted regarding the relationship of EBV with breast cancer, which have been accompanied by contradictory results. Some researchers reported the presence of the EBV in breast cancer (Labrecque *et al*., 1995; Glaser *et al*., 1998; Bonnet *et al*., 1999; Hemminki and Dong, 1999; Fina *et al*., 2001; Trablesi *et al*., 2008), while others have reported the lack of identification of the genetic material of this virus in samples of the breast tumor (Chu *et al*., 1998; Deshpande *et al*., 2002; Hermann and Niedobitek, 2003; Perrigoue *et al*., 2005). These contradictory findings may have been the result from the methods used in the diagnosis of the EBV genome (Lawson *et al*., 2006).

The hypothesis that the presence of EBV in the tumor tissues of the breast may have resulted from the infiltration of the lymphocytes infected with this virus was previously raised by a number of researchers. The studies conducted by Labrecque *et al*. (1995) showed clearly that EBV genetic materials existing mostly of cancerous cells and have benefitted from the lack of presence in the infiltrated lymphocytes. Also, it was confirmed that the EBV genetic materials in the epithelial cell available in the milk samples of healthy women are identifiable too (Junker *et al*., 1991). Because of the lack of documented information regarding the frequency of the Epstein Barr Virus in the benign and malignant tumors, the current study was designed and executed.

MATERIALS AND METHODS

Paraffin embedded samples of the breast carcinoma (N = 24) and fibroadenoma (N = 24) were collected from the pathology ward of Toos and Firozgar hospitals of Tehran, IRAN in 2011-2012. The carcinogenicity of the samples was diagnosed with the aid of the pathologist and based on the Richardson classification system.

Protocol of deparaffin of the samples: At first, ten 5μ cutting were provided from the paraffin blocks by the sterile microtome blade (N35) and transferred to sterile containers. In order for deparaffinization to occur, the samples were left in a xylene solution (Merck Germany) for 30 min. To dehydrate the samples, they were placed for 10 sec in ethanol solutions of 100, 80, 60 and 40% concentrate (Merck Germany), respectively. The tissue obtained was then transferred to sterile microtubes and stored under -20°C until the process of DNA extraction took place.

DNA extraction: Extraction of DNA from the tissue was implemented according to the instructions of the manufacturing company (Qiagene, Lot No: 11872534, Cat No: 51306). The purity of the extracted DNA was analyzed based on absorbance of the extracted DNA at 260 and 280 nm wavelengths by biophotometer (Eppendorf-Germany).

PCR method: Specific Primers produced by TAG Copenhagen (Denmark) were used to amplify the EBV gene. The sequences of forward and reverse primers were 5'-GTG TGC GTC GTG CCG GGG CAG CCA C-3' and 5'-ACC TGG GAG GGC CAT CGC AAG CTC C-3', respectively (Liloglou *et al.*, 1994).

Each reaction was performed in a total volume of 25 μL, which contained 14.5 μL of molecular biology-grade water (Sigma Aldrich Company LTD., USA), 2.5 μL of 10×PCR buffer (Promega, USA), 1 μL of 10 pM of forward and reverse PCR primers, 1 μL of 10 mM dNTPs (Promega, USA), 0.5 μL of smart *Taq* DNA polymerase (Promega, USA), 1 μL of 50 mM $MgCl_2$ (Promega, USA) and 5 μL of DNA template. The negative control tube contained the same PCR reagents as above but had 5 μL of water substituted for the DNA template. PCR amplification conditions on thermocycler (Biorad-Germany) were as follows: 94°C for 5 min, followed by 35 cycles of 95°C for 1 min, 52°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 5 min. An aliquot of all PCR products was run on a 1.5% (w/v) agarose gels with a 100 bp DNA ladder (Fermentas-Russia) and electrophoresed at 75 V for 40 min. The bands were visualized using ethidium bromide staining and photographed after UV treatment by a transilluminator (UV doc, England).

Statistical analysis: Chi square test was used to determine whether there was any significant difference between the frequency of EBV in the carcinoma and fibroadenoma samples and its relationship with the breast cancer (SPSS software 17).

RESULTS

The number of patients who suffered from the carcinoma based on age groups were: below 35 years old, 5 patients (21%), 35 to 55 years old, 12 patients (50%) and over 55 years old, 7 patients (29%). The average tumor size in 6 individuals (25%) was smaller than 2 cm and in 18 individuals (75%) the average tumor size was larger than 2 cm. Regarding the lymphatic glands under than arms, involvement of these glands was not diagnosed in 6 individuals (25%). But, involvement of the lymphatic glands was observed in 18 individuals (75%). Among the studied carcinoma samples, 22 samples of ductal carcinoma (92%), one sample of the lobular carcinoma (4%) and one sample of the mucinous carcinoma (4%) were diagnosed. Four individuals (16.6%) suffered from stage I malignant tumor, 9 individuals (37.5%) reached stage II and 11 individuals (45.9%) were diagnosed with a stage III (Table 1).

Regarding the demographic information of patients afflicted with fibroadenoma, only the age of the individuals was available. 17 individuals (71%) suffering from fibroadenoma were in the age group under 35 years and 7 individuals (29%) were in the age group of 35-55 years.

In order to identify the DNA of virus in the tissue samples, the PCR technique was used. The presence of a 375 bp band indicates the presence of EBV DNA in the sample (Fig. 1). In summary, four samples (16.6%) were positive from the view point of the presence of EBV among the 24 patients. One individual was in the age group under 35 years, two individuals were in the age group of 35 to 55 years and one individual was over 55 years old. Each of the 4 patients infected with the virus also suffered from the ductal carcinoma and average tumor size was larger than

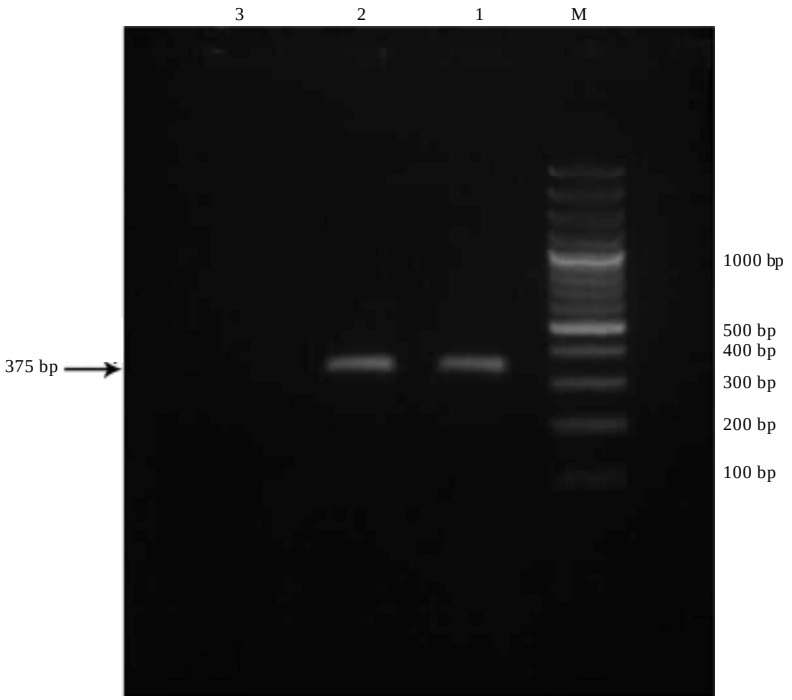

Fig. 1: EBV amplification products analyses in a 1.5% agarose gel stained with ethidium bromide, the presence of a 375 bp band indicates the presence of EBV DNA in the sample, Lane M: Marker size 100 bp, Lane 1-2: Amplified EBV DNA, Lane 3: Negative control

Table 1: Demographics of the breast cancer

Variable	Frequency
Age, years	
<35	5
35-55	12
>55	7
Tumor size (cm)	
<2	6
≥2	18
Tumor grade (SBR)	
I	4
II	9
III	11
Histology	
Ductal	22
Lobular	1
Mucinous	1
Nodal status	
Positive	6
Negative	18

The No. of specimens for evaluation = 24, SBR: Scarff, Bloom and Richardson

2 cm. Involvement of the lymph was positive in 1 individual (25%). Two individuals infected with virus suffered from stage III while stage I was diagnosed in one patient and stage II was diagnosed in another patient. Out of 24 samples of fibroadenoma, one sample (4.1%) possessed the genome

for this virus with this patient placed in the age group under 35 years. The statistical analysis did not show a significant relationship between the frequencies of this virus in the samples studied.

DISCUSSION

Epstein Barr virus is a prevalent virus so that 90% of the world's human population is infected (Gratama and Emberg, 1995). Infection resulting from this virus in developing countries occurs predominantly during childhood, while this condition is observed in teenagers (Moghim *et al*., 2007). The hypothesis exists that the infection resulting from EBV can create various cancers (Lawson *et al*., 2006). For example, Burkitt lymphoma in Africa, a nasopharyngeal carcinoma in the people of the southeast and Hodgkin lymphoma in western societies are amongst those cancers attributed to these viruses (Irshaid *et al*., 2010; Nikakhlagh *et al*., 2010; Hsu and Glaser, 2000; Niedobitek *et al*., 2001). This research, which was conducted to determine the frequency of EBV on the benign and malignant tumor, leads to the identification of this virus in each of the two kinds of tumors. Its prevalence in the ductal carcinoma and fibroadenoma was reported 16.6 and 4.1%, respectively. The frequency of EBV has been confirmed in a high percentage of patients suffering from breast cancer over the last decade. For example, Fina *et al*. (2001), Murray *et al*. (2003), Xue *et al*. (2003), Tsai *et al*. (2005) and Arbach *et al*. (2006) confirmed the presence of this virus in 32, 21, 40, 45 and 46%, breast tumors, respectively.

While the failure to identify the virus has been reported by a large number of researchers, including Gaffey *et al*. (1993), Lespagnard *et al*. (1995), Glaser *et al*. (1998), Chu *et al*. (1998), Dadmanesh *et al*. (2001), Deshpande *et al*. (2002) and Perrigoue *et al*. (2005), these contradictory results could be from the usage of different diagnosing techniques or usage of the paraffined fix tissues or new tissues.

Most of the studies conducted, which have been able to show the presence of the virus's genome in tumor tissues, used the PCR technique. Compared to PCR, the technique such as *in situ* Hybridization and Immunohistochemistry often reported the negative results (Lawson *et al*., 2006).

Another reason for the failure to identify the EBV in breast tumors, conducted by Murray *et al*. (2003) and Perrigoue *et al*. (2005) has been attributed to the presence of EBV in the infiltrated lymphocytes in breast tumors, which this hypothesis was denied by a number of exact studies. Identification of the genetic material of EBV in about half of the milk samples of the healthy women is a confirmation of the presence of this virus in the epithelial cells (Junker *et al*., 1991).

Although, the expression of the EBV genetic materials in breast tumor compared to the tumors such as nasopharyngeal carcinoma or Burkitt lymphomas vary less, the presence of the virus in the epithelial cells can be a cofactor for the formation of the cancer (Lawson *et al*., 2006).

CONCLUSION

The results obtained by this study show the presence of EBV in both malignant and benign tumors of breast and it seems that prevention from infection with this virus can play a more effective role in the reduction of the affliction with breast tumor. However, further studies are needed to confirm the role of EBV in tumor formation.

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