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### Frequency of Cytomegalovirus (CMV) in benign and malignant tumors

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#### ABSTRACT

Cytomegalovirus (CMV) is a widespread pathogen that is found in milk, saliva, urine, cervical secretions, and semen fluid. It is supposed that CMV is a risk factor for breast cancer. The aim of this study was to determine the frequency of CMV among benign and malignant breast tumors. Paraffin embedded breast carcinoma (n=24) and fibroadenoma (n=24) samples were collected from Toos and Firoozgar hospitals in Tehran, Iran, during the year 2012. PCR technique was used to detect CMV genome in the samples following the DNA extraction. Out of 24 carcinoma samples, 2 (8.3%) were positive for CMV, while no CMV positive sample was found among fibroadenoma tumors. Statistic analysis showed no significant correlation between this virus and formation of either benign or malignant tumors of breast. The role of CMV in a number of human cancers has been shown during the past decade. This study shows no relationship between CMV infection and breast benign and malignant tumors. However, future studies are needed to better understand the role of this virus in formation of breast tumors.

#### 1. Introduction

Breast cancer is the uncontrolled division of cells, which can occur in different parts of breast including ducts, which transfer breast milk, milk producing tissue and in unglandular tissue. Breast cancer is the most common cancer among women (Parkin et al., 2005). Based on the report given by the National Cancer Institute of the United States, one out of every eight women will develop this cancer during their lifetime (Wong et al., 2002; Khorshid, 2011). Genetic elements such as mutation in BRCA1 and BRCA2 genes are considered as the main risk factor in breast cancer (Miki et al., 1994; Wooster et al., 1995). However, other risk factors

such as age, family history, early menstruation before age 13, late menopause after age 51, women who have never become pregnant or late pregnancy after 30, obesity, alcohol, and viral infections play important roles in this disease (Wooster et al., 1995; Rand et al., 2009). Many studies conducted during the past two decades have shown that viruses play a role in the development of breast cancer (Boulanger et al., 2001; Poiesz et al., 2001; Trablesi et al., 2008; Zaravinos et al., 2009; Irshaid et al., 2010; Nikakhlag et al., 2010; Eghbali et al., 2012a; Eghbali et al., 2012 b).

Cytomegalovirus, as a member of Herpes viridae family, causes constant infection in humans. CMV is the largest member of Herpes viridae with

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a 240 Kbp double stranded DNA, which encodes more than 200 proteins. Similar to other members of Herpesviruses, CMV capsid is coated by an envelope consisting of bilayer lipids and some proteins encoded by the virus (Ryan and Ray; 2004). CMV is mainly transmitted through blood and sexual intercourse (Odida and Schmauz, 1996).

Presence of cytomegalic cells with intranuclear inclusion bodies, which look like Owl's eyes under the light microscopy, are considered as the pathological key to confirm CMV infection in tissues. CMV prefers lymphocytes and mononuclear cells to infect; however, it can be found in almost any organ. Studies show that CMV can be considered as a co-factor in oncogenicity of cervical cancer (Yang et al., 2004a; Yang et al., 2004b). Other studies have also shown that CMV may play a role in thyroid cancer (Tsai et al., 2005). CMV is supposed to be a risk factor for breast carcinomas too (Richardson, 1997). This study aimed to investigate the presence and frequency of CMV in benign and malignant breast tumors.

## 2. Materials and Methods

### 2.1. Sample collection

Paraffin embedded breast carcinoma (n=24) and fibroadenoma (n=24) samples were collected from Toos and Firoozgar hospitals in Tehran, Iran, during the year 2012. Carcinoma and fibroadenoma samples were classified based on Richardson classification system by a pathologist.

### 2.2. deparaffinization

Few 5 µ slices were cut from paraffin embedded breast tumor samples by sterile microtome blades (N= 35) and were transferred into sterile 3ml tubes. Tissue sections were treated by xylene (Merck, Germany) for 30 min to deparaffinate. To dehydrate, samples were soaked into 40%, 60%, 80%, and absolute ethanol each for 10 sec. Samples were thereafter transferred into sterile microtubes and stored at -20°C.

### 2.3. DNA extraction

Extraction of DNA was carried out by Miniprep DNA Extraction Kit (Qiagene, Lot No:11872534, Cat No: 51306) based on manufacturer's

instruction. The concentration and purity of DNAs were determined by biophotometer (Eppendorf-Germany) at 260 nm a 260/280 ratio, respectively.

### 2.4. Human beta-globin gene amplification

Human beta-globin gene was co-amplified with the target fragment, as an internal amplification control, using the following primers: b2-microglobulin-F: 5'-TCC AAC ATC AAC ATC TTG GT-3' and b2-microglobulin-R: 5'-TCC CCC AAA TTC TAA GCA GA-3'. Each reaction was performed in a total volume of 25 µl, which contained 13 µl of molecular biology-grade water (Sigma Aldrich Company LTD., USA), 2.5 µl of 10×PCR buffer (Promega, USA), 1 µl of 10 pmol 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<sub>2</sub> (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 94°C for 50 S, 54°C for 45 S and 72°C for 40 S, 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).

### 2.5. CMV gene amplification

Specific primers produced by TAG Copenhagen (Denmark) were used to amplify the CMV gene. The sequences of forward and reverse primers were 5'-GTC ACC AAG GCC ACG ACG TT-3' and 5'-TCT GCC AGG ACA TCT TTC TC-3', respectively (Zaravinos et al., 2009).

Each reaction was performed in a total volume of 25 µl, which contained 13 µl of molecular biology-grade water (Sigma Aldrich Company LTD., USA), 2.5 µl of 10×PCR buffer (Promega, USA), 1 µl of 10 pmol 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<sub>2</sub> (Promega, USA) and

5 µl of DNA template. The negative control tube contained the same PCR reagents as mentioned above but had 5 µl of water substituted for the DNA template. Cycling conditions was set up as follows: 94°C for 1 min as initial denaturation, 94°C for 50 s, 64°C for 50 s, and 72°C for 50 s for 30 cycles, and 72°C for 2 min as final extension.

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).

### 2.6. Statistical analysis

Statistical analysis was carried out using SPSS (Chicago, IL, USA) software version 17.0 for Microsoft Windows®. The results were processed statistically using the Chi square test to compare the frequency of CMV among carcinoma and fibroadenoma samples and its association with breast cancer.

## 3. Results

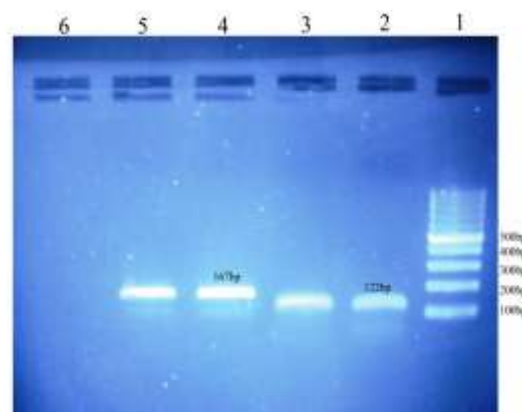
The number of patients who were suffering from the carcinoma based on age groups was 5 patients (21%) below 35 years old, 12 patients (50%) between 35 to 55 years old, and 7 patients (29%) over 55 years old. The average tumor size in 6 individuals (25%) was smaller than 2 cm and in 18 individuals (75%) it was larger than 2 cm.

Regarding the involvement of the lymphatic glands under the arms, 18 patients (75%) were diagnosed with no involvement, while 6 patients (25%) showed involvement in these glands. Among the studied carcinoma samples, 22 samples (92%) were diagnosed with ductal carcinoma, one sample (4%) with lobular carcinoma, and one sample (4%) with mucinous carcinoma. Moreover, the malignant tumors were diagnosed at stage I, stage II, and stage III in 4 (16.6%), 9 (37.5%), and 11 (45.9%) patients, respectively.

Regarding the demographic data of patients who were suffering from fibroadenoma, only the age of the patients was available. Based on this data, 17 patients (71%) were under 35 years old, while 7 patients (29%) were between 35 to 55 years old. No patient was over 55 years old.

PCR technique was used to identify the DNA of CMV in the tissue samples. The amplified fragments of human beta-globin gene and viral DNA were 122 bp and 167 bp, respectively (Fig 1).

As the result, out of 24 carcinoma samples, only 2 (8.3%) were determined as positive for CMV; each of the 2 patients infected with the virus were in the age group of 35 to 55 years. Both patients infected with the virus were suffering from the ductal carcinoma. None of the infected patients showed lymphatic glands involvement. Two individuals infected with virus suffered from stage II. Moreover, regarding the tumor size, 1 sample was larger than 2 cm in diameter, and 1 samples were below 2 cm. Regarding the fibroadenoma samples, no sample was infected with CMV. The statistical analysis showed no significant relationship between CMV infection and increased risk of both carcinoma and fibroadenoma in samples studied ( $P=0.149$ )



**Figure 1.** Amplification products analyses in a 1.5% agarose gel stained with ethidium bromide. Lane 1: size marker 100bp, Lane 2 and 3 human beta-globin gene, Lane 4 and 5 viral DNA and Lane 6 negative Control.

## 4. Discussion

In addition to the persistent infection in humans, CMV causes severe infections in newborns and those with immune system deficiency. In developing countries, most of the people are infected with CMV during their childhood and almost 100% of adults show serological evidence of prior CMV infection, while this rate for the adults in developed countries is 50%. Congenital infection of fetus with CMV during the pregnancy is a major health burden. About 15% of newborns show

evidence of CMV infection at birth (Michaelis et al., 2009; Lopo et al., 2011).

Many studies performed during the past decade have revealed that Human CMV (HCMV) plays role in a number of human malignancies including glioma, colorectal, prostate, and skin tumors (Michaelis et al., 2009). Evidences show that gene products of this virus can modulate the oncogenic properties of human cells in vitro. These gene products cause mutation in DNA, arrest apoptosis pathways, inhibit cell immunity, and impair tumor suppressor proteins. Therefore, cells infected with this virus are prone to neoplasia due to genetic instability and immunologic deficiency (Boldogh et al., 1994; Shen et al., 1997; Doniger et al., 1999).

This research, which was conducted to determine the frequency of CMV on the benign and malignant tumor, leads to the identification of this virus in malignant tumors. Its prevalence in the ductal carcinoma and fibroadenoma was reported 8.3% and 0%, respectively.

In a study carried out by Richardson and his colleagues, a relationship was shown between high levels of anti-CMV IgG antibody and breast cancer in women. The level of anti-CMV IgG antibody was measured in the plasma of 208 women who were suffering from breast cancer and 169 healthy women. The result showed that CMV infection can be considered as a risk factor for breast cancer (Richardson et al., 2004).

In the study performed by Harkins and his colleagues in 2010 on the frequency of HCMV in the normal and neoplastic epithelium of breast, this virus was detected in 63% of normal epithelium and 97% of ductal carcinomas (Harkins et al., 2010).

This virus has been isolated from the milk and normal epithelium of breast. Many evidences have confirmed that HCMV plays role in tumor formation through modulation of intra-cellular signaling pathways.

## 5. Conclusion

The results obtained by this study show the presence of CMV in malignant 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 CMV in tumor formation.

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