RESEARCH ARTICLE

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Human Cytomegalovirus DNA among Women with Breast Cancer

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Abstract

Breast cancer is the most common cause of death among women worldwide. Although there are many known risk factors in breast cancer development, infectious diseases have appeared as one of the important key to contribute to carcinogenesis formation. The effects of Human Cytomegalovirus (*HCMV*) on women with breast cancer has been recently studied and reported. To contribute to this research trend, this study was conducted to evaluate the association between *HCMV* and the women with breast cancer. **Objective:** This experiment aimed to evaluate *HCMV* DNA in women with breast cancer in Ahvaz city, Iran. **Materials and Methods:** A total of 37 formalin fixed paraffin embedded tissues of the patients with ductal breast carcinoma and 35 paraffin embedded tissues of the patients with fibro adenoma as control group were collected. The deparaffinization of all the samples were carried out and the DNA was extracted. Initially, the PCR test was carried out to detect beta –globulin DNA as an internal control. For those samples positive for beta –globulin DNA, Polymerase Chain reaction (PCR) was used to detect *HCMV* for the tests and control samples. **Results:** Among 37 ductal breast carcinoma, 20 (54.04%) cases were proved positive for *HCMV* DNA by PCR. While among the 35 control group (fibroadenoma), 10 (28.57%) cases were positive for *HCMV* DNA (P >0.028). The prevalences of *HCMV* DNA among the age groups 30-39, 40-49 and >50 years were 7 (72.22%), 9 (69.23%), 4 (57.14%), respectively (P=0.066). A high frequency of *HCMV* DNA was detected in tumor grade III, 13/18 (58.33%) compared with tumor grade II, 7/19 (36.84%) (p=0.044). A high frequency of 16/24 (66.66%) of *HCMV* DNA was found in invasive ductal breast cancer compared with 4/13 (30.76%) *HCMV* DNA in situ (P<0.028). **Conclusion:** A high prevalence of 54.05% *HCMV* was found among the patients with ductal carcinoma. The percentages of the high prevalence of *HCMV* among age group (40-49) years, tumors grades, and invasive stage were (69.23%), (58.33%), (66.66%), respectively. Further study of *HCMV* in the latency phase in patients with ductal carcinoma would be necessary to extend our knowledge.

Keywords: Human cytomegalovirus- breast cancer- ductal carcinoma- Polymerase Chain Reaction

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Introduction

Breast cancer is a leading cause of death among women worldwide. There are several risk factors for breast carcinoma such as early age of menarche, late age of menopause, a positive family history of breast cancer, hormone replacement therapy, age, sex, and nulliparity (Ban et al., 2014; Jemal et al., 2011; Anders et al., 2009). The prevalence of breast cancer has been frequently reported in Iran (Sajad et al., 2009; Mohammadizadeh et al., 2014; Karimi et al., 2016; Mohammadizadeh et al., 2017). So far, investigations have implied a number of different viral infections associated with breast cancer, including bovine leukemia virus (Buehring et al., 2015). Human mammary tumor virus (Melana et al., 2010), human papillomavirus (Heng et al., 2009), Epstein–Barr virus (EBV) (Joshi et al., 2009; Fawzy et al., 2008; Hachana et al., 2011), polymavirus (Antonsson et al., 2012) and human cytomegalovirus (*HCMV*) (Karimi et al., 2016; El-Shinawi et al., 2013; Richardson et al., 2015; Mohammadizadeh et al., 2017). Although the role of these viruses in the breast cancer are arguable, the evidence of molecular epidemiological suggests an association between *HCMV* and breast cancer. *HCMV* is a β-herpesvirus that affects 70–90% of the world population, causing general, acute, persistent, or lifelong

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latent infection (Goodrum et al., 2012). *HCMV* infections are typically subclinical and serious diseases which occur chiefly in immune-compromised individuals (Dziurzynski et al., 2011). Overall, *HCMV* serostatus has not been positively associated with breast cancer. However, women with breast cancer were found to have higher mean *HCMV* IgG levels in an Australian case–control study suggesting that they might have afflicted a recent infection (Richardson et al., 2004). Although *HCMV* proteins and DNA have been detected in breast tumor tissue, *HCMV* is not typically considered as an oncogenic virus (Michaelis et al., 2009). *HCMV* can promote many classic signs of cancer, such as cell cycle dysregulation, inhibition of apoptosis, increased migration and invasion, and immune evasion (Sanchez et al., 2008). *HCMV* has been associated with other malignancies, including glioblastoma (Soroceanu et al., 2011), medulloblastoma (Baryawno et al., 2011), colon cancer (Tafvizi et al., 2014), prostate cancer (Samanta et al., 2003) and Hodgkin and Non-Hodgkin Lymphoma (Hamide et al., 2017). Individual *HCMV* gene products can have profound effects on cell growth, such as immediate early proteins (*IE1*) and *IE2*, which are known to stimulate entry into S phase (Castillo et al., 2002). *IE1* expression was observed to increase the growth rate of glioblastoma cells in culture, suppress p53 and Rb tumor suppressor activity, and stimulate PI3K/Akt signaling (Cobbs et al., 2008). *IE1* was detected in breast tumor tissue (Harkins et al., 2010; Taher et al., 2013). Another *HCMV* gene, US28 displays constitutive signaling activity, and cells expressing US28 are highly invasive and form tumors in nude mice (Soroceanu et al., 2011; Maussang et al., 2009). US28 was shown to induce vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX2), and STAT3 activation through up-regulation of IL-6 (Maussang et al., 2009; Slinger et al., 2010). The *HCMV UL111A* gene encodes cmvIL-10, a viral cytokine which is secreted from infected cells (Jones et al., 2002). Extensive immunosuppressive properties of cmvIL-10 was found to down regulate class I and II MHC, and inhibit the dendritic cells maturation (Slobedman et al., 2009; Chang et al., 2009; Avdic 2014). Engagement of the IL-10 receptor by cmvIL-10 leads to activation of STAT3 which is commonly activated in breast cancer cells (Banerjee et al., 2016). It was found that STAT3 activation is associated with poor prognosis in ovarian cancer and is considered as a key factor in metastasis formation (Zhang et al.,2010). With the aforementioned properties of *HCMV* and its association with different human cancers, this study was conducted to evaluate the *HCMV* DNA in the formalin-fixed paraffin-embedded tissues of the patients with ductal breast carcinoma in Ahvaz city. Ahvaz city is capital of Khuzestan province with the population of 1.5 million people, located in the southwest of Iran.

Materials and Methods

Fifty blocks of the formalin-fixed paraffin-embedded tissue blocks of ductal breast cancer and forty three fibroadenoma as a control group were collected from the archive of Imam Khomeini Hospital, Ahvaz, Iran during 2006-2014. The diagnostic accuracy of ductal breast carcinoma were approved by a pathologist. The patients ages were between 40 and 59 years with the mean age of 55±8 years. The sections of 10 µm thickness were prepared from each sample and stored at 4°C until tests performance .

1-Deparaffinization: Deparaffinization was done by xylene and ethanol (Germany, Merk). Initially, all the specimens were placed in microtubes then xylene was added and kept at 45C° for 15 min followed by centrifuge at 14,000 rpm for 1 minute. This stage was repeated again. The supernatant was discarded and 1ml absolute ethanol was added to precipitate. It was stored at the room temperature for 10 min and centrifuged again at 14,000 rpm for 1 minute. The supernatant was discarded. This process was repeated by adding 70% ethanol, followed by the same condition. Finally, supernatant was discarded and all microtubes were placed at 65°C for 5 min to vaporize the ethanol residue and the pellet was used in DNA extraction (Habibian et al., 2013).

2-DNA extraction: High pure PCR template preparation kit (Roche, Germany, code No: 11796828001) was applied for the extraction of DNA, according to the manufacturer's instruction. The extracted DNA was stored at -70°C until PCR amplification.

3-PCR amplification: All the extracted DNA samples were initially subjected to PCR with consensus primers PCO3/PCO4 (β-globin) to confirm the quality of the extracted DNA (used as an internal control). The following primers (PCO3: 5́ACACAACTGTGTTCACTAGC/PCO4: 5́CAACTTCATCCACGTTCACC with PCR product of 110 bp (Shahab et al., 2015). The following primers conserved for the GB region of HCMV included, F primer 5́- TCTGGGAAGCCTCGGAACG -3 (1,043-1,062) and R primer 5- GAAACGCGCGGCAATCGG-(1,621-1,604). was used to detect *HCMV*. The first round of PCR was performed in 25µl mixture, containing 10µl of extracted DNA, 2.5µl PCR buffer 10X (Roche),0.5µl dNTP 10mM (Roche), 1U Taq Polymerase (Roche), 1µl(20µM) of each primer sequence, D/W up to 25µl was subjected to thermocycler (Techne TC-5000, UK) (Gilbert et al.,1999). The products of 581bp indicating positive reaction.

4-Gel electrophoresis: The second round of PCR product was separated on a 2% agarose gel and developed by Safe Stain under voltage at 100V. The result was seen under ultra violet in transilluminator. The sizes of bands were compared with 100bp Ladder (Fermentas) which was placed on the well as an indicator.

To confirm the results of PCR and to determine genotyping randomly, 5 positive PCR products were selected and sequenced (Bioneer company, South Korea). The sequences were blasted using available databases.

Statistical analysis

The obtained results were analyzed by the version 17 of SPSS software and the role of age and sex on positive cases were surveyed by Fisher`s exact and Chi square test.

Results

Among 37 ductal breast carcinoma, 20 (54.04%) cases

were positive for *HCMV* DNA by PCR. On the other hand, among the 35 control group (fibroadenoma), 10 (28.57%) cases were positive for *HCMV* DNA (P >0.028). The prevalence of *HCMV* DNA among the age groups 30-39, 40-49 and >50 years was 7(18.91%), 9 (69.23%), 4/7 (10.81%) respectively (P=0.066). High frequency of *HCMV* DNA have been found in tumor grade III13/18 (72.22%) compared with tumor grade II7/19 (36.84%) (p=0.044). A high rate of 16/24 (66.66%) *HCMV* DNA was found in invasive ductal breast cancer in comparison with the results 4/13 (30.76%) *HCMV* obtained in situ (P<0.028) (Table 1).

Discussion

Although, the association between *HCMV* and cancer is arguable, there are documentations which reveal that progression of some tumors could be intensified by the proteins named *US28*, *pp65*, *IE1*, encoded by *HCMV* genes during the phase of latent infection (Soroceanu et al., 2011; Maussang et al., 2009; Cai et al., 2016; Lucas, 2011). *PP65* is the most abundant virion protein and non-infectious viral particles that are assembled during active infection (Libard et al., 2014). Besides, the detection of IE1mRNA indicates the presence of activation or reactivation of *HCMV* infection (Harwardt., 2016). The IE protein is detected in breast tumor tissue (Harkins et al., 2010; Taher et al., 2013; Mohammadizadeh et al., 2017). However, the association and expression of *US28*, *pp65* and *IE1* genes were not evaluated in the present study, which requires further investigations. Additionally, there are some factors involved in the latency of *HCMV*. These proteins are UL133-UL138, encoded by *HCMV* genes and involved in regulating latency, viral immune escape and cell tropism (Petrucelli et al., 2012; Montag et al., 2011; Hamide et al., 2016). The detection of *HCMV UL-133,-138* genes were not carried out in the presence study.

Table 1. Profile of Patients with Breast Cancer Type Ductal Carcinoma

Category	HCMV positive	HCMV negative	p-value
Ages			
<20	0/5	5/5	0.066
30-39	7/12 (58.33%)	5 (41.66%)	
40-49	9/13 (69.23%)	4 (30.76%)	
<50	4/7 (57.14%)	3 (42.85%)	
Ductal carcinoma			
No:37	20/37 (54.05%)	17/37 (45.94%)	0.028
Adeno			
Fibroma (No:35)	10/35 (28.57%)	25 (71.42%)	
Tumor grade			
Grade II	7/19 (36.84%)	12 (63.15%)	0.044
Grade III	13/18 (72.22%)	5 (27.77%)	
Breast cancer			
Invasive N=24	16/24 (66.66%)	8 (33.33%)	0.028
In situ N=13	4/13 (30.76%)	9 (69.23%)	

Table 1 shows that the distribution of HCMV among the age-group was not significant (*p=0.066*), while it was found significant among the ductal carcinoma and fibroma (*p=0.028*), tumor grade II and III (*p=0.044*), and invasive and in situ (*p=0.028*).

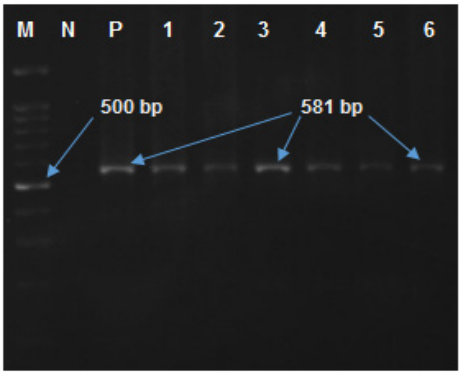

Figure 1. Lane M, molecular size maker, 100 bp DNA ladder; lane N, negative controls; Lane P, positive control; Lane 1-6, amplified products (581bp) on agarose gel electrophoresis

In the presence study, a high prevalence of 54.04% *HCMV* DNA was found in patients with ductal breast cancer while 28.57% of control group were positive for *HCMV* DNA. The status of *HCMV* detection in the latency phase in patients with ductal breast cancer was not clear but 20 (54.04%) patients with ductal breast carcinoma appeared positive for *HCMV* DNA by PCR. On the other hand, among the 35 control group samples (fibroadenoma), 10 (28.57%) cases were positive for *HCMV* DNA (P >0.028.). In accordance to our findings, (Karimi et al., 2016) detected *HCMV* DNA in 26/50 (58%) samples of invasive breast carcinoma by using the nested-PCR method in Sanandaj city, Iran.

Taher et al., (2013) from Sweden demonstrated *HCMV* IE protein expression in 100% of 73 breast cancer samples using the IHC method and real-time PCR. Harkins et al., (2010) in the United States also evaluated the surgical biopsy specimens of 38 normal breast samples, 39 breast carcinoma samples, and paired normal breast tissue from 21 breast cancer patients, and demonstrated a higher expression of *HCMV* immediate early (IE), early and late (E/L) and late (L) antigens in breast cancer 31/32 (97%) compared to normal breast epithelium 17/27 (63%).

El-Shinawi et al., (2013) from Egypt also reported a significant association between *HCMV* and breast cancer with higher serum levels of *HCMV* IgG in 82% of 28 patients with inflammatory breast carcinoma compared to 65% of 49 patients with non-inflammatory breast carcinoma.

On the contrary, several studies have not spotted any relationship between *HCMV* and breast cancer. Utrera-Barillas et al., (2013) evaluated 27 breast cancer specimens and 20 fibroadenoma samples by quantitative PCR and reported no significant association between *HCMV* and breast cancer development in Mexico. Richardson et al., (2015) from New Zealand also evaluated the CMV IgG levels in plasma (by the enzyme immunoassay method) and checked *HCMV* DNA in 70 tumor samples using the quantitative PCR method and found no relationship between *HCMV* and breast cancer.

In conclusion, this research documented a high prevalence of 54.05% for *HCMV* DNA among the patients with ductal carcinoma. In detail, high prevalence rates of *HCMV* DNA among the patients' group (40-49) years (69.23%), tumors grades (72.22%), and invasive stage (66.66%) were detected.

In conclusion, further research is needed to investigate the *HCMV* role in the latency phase in patients with ductal carcinoma as the role of *HCMV* in the latency phase and it association with ductal carcinoma could not be justified by this experiment. To do this, the expression of *UL-133-138*, *US28*, *pp65* and *IEI* genes needs to be evaluated. To manage and treat individuals with breast cancer prior to chemotherapy, the detection of *HCMV* by PCR or Real time PCR should be implemented.

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Authors' Contributions

Study concept and design: Manoochehr Makvandi, Alireza Samarbafzadeh; acquisition of data: Payman Sepahv; analysis and interpretation of data:

Manoochehr Makvandi and Payman Sepahvand; drafting of the manuscript: Payman Sepahvand; critical revision of the manuscript for intellectual content: Manoochehr Makvandi; statistical analysis: Ahmadi Angali Kambiz and Payman Sepahvand; administrative, technical, and material support: Payman Sepahvand, Niloofar Neisi, Abdolhassan Talaei-Zadeh, Nastarn Ranjbari, Nilofar Nisi. Azarakh Azaran, Shahram Jalilian, Mehran Varnaseri; study supervision: Manoochehr Makvandi and Alireza Samarbafzadeh.

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The authors have no financial interest related to the material in the manuscript.

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