Introduction

Breast cancer is a leading cause of death among women worldwide. There are several risk factors for 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 fibroadenoma 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 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
Peyman Sepahvand et al
Asian Pacific Journal of Cancer Prevention, Vol 20

2276 Asian Pacific Journal of Cancer Prevention, Vol 20

HCMV infections are typically subclinical and serious diseases which occur chiefly in immune-compromised individuals (Dziuryznski 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., 2011). 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 45°C 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: 5’ACAACACTGGTGTCTAGCC/PCO4: 5’CAACTTCATCCACGTCTACC with PCR product of 110 bp (Shahab et al., 2015). The following primers conserved for the GB region of HCMV included, F primer 5’- 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),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 III 13/18 (72.22%) compared with tumor grade II 17/19 (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 IE1 mRNA indicates the presence of activation or reactivation of HCMV infection (Harvardt., 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.

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.

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 No:24            | 16/24 (66.66%)| 8 (33.33%)    | 0.028   |
| In situ No: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).
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 IE1 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 Samarbazfzadeh; acquisition of data: Payman Sepahv; analysis and interpretation of data: Manoochehr Makvandi and Payman Sepahv; drafting of the manuscript: Payman Sepahv; critical revision of the manuscript for intellectual content: Manoochehr Makvandi; statistical analysis: Ahmad Angali Kambiz and Payman Sepahv; administrative, technical, and material support: Payman Sepahv, Niloofar Neisi, Abdolhassan Talaei-Zadeh, Nastarn Ranjbari, Nilofar Nisi. Azarakh Azaran, Shahram Jalilian, Mehran Varnaseri; study supervision: Manoochehr Makvandi and Alireza Samarbazfzadeh.

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