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Relative Frequency of Cytomegalovirus (CMV) in tissue samples of women with breast cancer in Sanandaj, Iran.

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Abstract: Despite the researchers' efforts, the cause and development of breast cancer is still incompletely understood. Currently, in some reports, human cytomegalovirus has been referred as a risk factor for breast cancer**.** This study aimed to determine relative frequency of cytomegalovirus in tissue samples of women with breast cancer in Sanandaj County. In this study, to determine the relative frequency of the human cytomegalovirus (CMV) 50 formalin-fixed tissues of breast cancer, which all were invasive ductal carcinoma, were studied using the nested-polymerase chain reaction. In 26 cases of breast cancer tissues (26/50), human cytomegalovirus was detected. Seventeen cases of breast cancer tissues were in a moderately differentiated stage, and nine cases had poor-differentiated stage tissues that were positive for viral DNA. At older ages (>45 years) the prevalence rate of human cytomegalovirus DNA was higher, but no significant association was seen (p=0.16)**.** In general, due to the high prevalence of the DNA of human cytomegalovirus (58%), in this study it is assumed that human cytomegalovirus (HCMV) has a contributing role in breast cancer; although more study is required to clearly define its part in this type of cancer.

Key words: Human Cytomegalovirus (HCMV), Breast Cancer, Nested PCR, ductal carcinoma

Introduction

Breast cancer in women worldwide is considered as the most widespread disease and a most important etiology of mortality (1, 2) and during 2012 greater than 226,870 new cases of invasive breast cancer were recorded (3). Cases of breast cancer from the second decade of life were detected and from the ages of 40 to 54 years there was a peak in the incidence rates. There were highest of cases in highly developed second, third, and fourth stages. In the solid malignancy development, metastasis has been considered frequently as a last and mortal step, and for a number of these steps, certain tissues can take into account as the molecular requirements (4). A number of risk factors, for example, gender, age, obesity, estrogen level, and breast cancer family history facilitates an increase in breast cancer incidence rate (2, 5). However, these risk factors may be missing in a total of 50–80% of the patients; consequently, in the recent years, there has been an improved attention in ascertaining additional risk factors playing a role in the disease (2). In accordance with the recent studies, cancer, including breast cancer may be caused by chronic viral infection interference (6,7). Some viral infections, including human herpesvirus 4 (8), mouse mammary tumor virus (MMTV) (9), human papillomavirus (HPV) (10) and newly, human cytomegalovirus (HCMV) (11) and human herpesvirus-8 (HHV-8) in breast cancer were in a positive association with lower relapse-free time and overall survival (10). According to growing evidence during the past 10 years, there is an association between HCMV with numerous human malignancies, including malignant glioma, colorectal carcinoma, prostate and skin cancers; furthermore, HCMV gene products can modify the oncogenic properties of cells in vitro (12–17). A common human pathogen, *i.e.* HCMV, which belongs to the β-herpesvirus family, infects nearly 70–90% of the world's population. After primary infection and reactivates sporadically this pathogen remains latent in its host (2). A number of mechanisms could be extant about the CMV ability in initiating and progressing breast cancer. Initially, it was revealed that HCMV gene products influence regulation of cell cycle, and inhibit apoptosis; moreover, they activate angiogenesis and metastatic phenotype and, cause the increased rate of mutation, thus overlapping with all the established hallmarks of cancer cells (18). Furthermore, HCMV displays immunosuppressive properties, leading to the tumor cells escape from immune surveillance mechanisms (19). Regardless of the absence of evidence concerning the presence of HCMV in human breast glandular tissues, breast glandular epithelium is considered as a probable reservoir for persistent infection of HCMV in humans. This paper aimed to determine the HCMV infection frequency in women with breast cancer in the city of Sanandaj, located in Kurdistan Province, Iran.

Materials and Methods

Study group: All specimens were prepared in the Pathology departments of Beassat and Tohid Hospitals of Sanandaj, and examined by a pathologist, and then, they were used for DNA

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extraction and PCR analysis. In the study the specimens included 50 formalin-fixed tissues of breast cancer, all of them of invasive ductal carcinomas (IDC). Of 50 IDCs, well differentiated, moderately differentiated, and poorly differentiated IDCs were five (20.8%), 29 (58%), and 16 (32%), respectively. In this study the trials were started in following four steps:

Deparaffinization: The specimens were deparaffinized using xylene and ethanol (Germany, Merk). Initially, all collected specimens were placed in microtubes; subsequently, xylene was added to them and the tubes were reserved at 45°c for 15 minutes and after that, they were centrifuged at 14000 RPM. This leg was repeated once more. The supernatant was hand-me-down, 1ml absolute ethanol was added to the precipitate, and then it was kept at the room temperature for 10 minutes and was centrifuged again at 14000 RPM for one minute. After casting off the supernatant, 1ml of 70% ethanol was added and exactly like what was done with absolute ethanol, the mixture was placed in the room temperature for 10 minutes and centrifuged at 14000 RPM. After this step, the supernatant was discarded again and lastly all the microtubes were subjected to the temperature of 65°c for five minutes in order to turn the remaining ethanol into vapor, and then the sediments were used in DNA extraction (20).

DNA extraction: According to the manufacturer's instructions the entire cellular DNA was extracted from the samples using the high pure PCR template preparation kit (Roche, Germany). To enhance the outcome, the extracted DNA was stored at -70°c until PCR amplification **(20).**

Verification of DNA extraction: Using amplification of the β-globin the whole samples were examined for DNA veracity. The primer sequences were as follows: PCO3/PCO4 (PCO3: 5´ ACA CAA CTG TGT TCA CTA GC /PCO4: 5´ CAA CTTCAT CCA CGT TCA CC) (21). The PCRs were performed with the final volume of 25µl, made up of 12.5µl of the Amplicon master mix, 0.5µl of forward and reverse primers and 10µl of DNA template. In addition, amplification was performed in the thermocycler. After an initial denaturation phase at 95°C for three minutes, 45 cycles programmed were along these lines: denaturation phase at 95°C for 35 seconds, annealing phase at 55°C for 45 seconds, primer extension at 72°C for 40 seconds and final extension phase at 72°C for five minutes. PCR products were determined by visualization of amplicons on 2% agarose gels stained with Safe Stain(22).

Nested PCR amplification: The PCR amplification's first round of was carried out in a 25µL reaction volume, including 12.5µl of Amplicon master mix, 0.5µl of forward and reverse primers and 10µl of each genomic DNA sample. Moreover, the sequences of oligonucleotides primers were as follows: 5'TCCAACACCCACAGTACCCGT-3'and 5'CGGAAACGATGGTGTAGTTCG-3'(23).

The program of PCR was carried out along these lines: 1) pre-denaturation at 95°C for five minutes, one cycle; 2) denaturation at 94°C for 35 seconds, annealing at 50°C to for 40 seconds, extension at 72°C for 35 seconds, 20 cycles; and 3) post-extension at 72°C for 5minutes, one cycle. The second-round PCR amplification was performed via 12.5µl of the Amplicon master mix, 0.5µl of forward and reverse primers and 5µl of the first-round PCR products as templates in 25µL reactions(22). The sequences of the inside primer pair were: 5'GCCCGCCGCGGCAGCACCTGGCT-3' and 5'GTAAACCACATCACCCGTGGA-3' (23). In the last part of the amplification, 6 µL of the PCR products were analyzed on 2% agarose gel. It was anticipated that the resultant product is a 174-bp fragment(22).

Results

In this study, the mean age of the patients was 46.18 years (minimum: 25 years old; and maximum: 55 years old). The studied tissues were 50 invasive ductal breast carcinoma categorized in one of the triple stages: 1) well differentiated (G1); 2) moderately differentiated (G2), and 3) poorly differentiated (G3). Based on the histology grade and age of the patients with breast cancer, distribution of HCMV is listed in table1. The β-globin primer was used to conduct a PCR in order to extract DNA from the tissues. After verification of the 110-bp fragment, the samples were selected for the nested-PCR. Considering the extracted DNA quality and quantity, 110-bp fragments had been proliferated in all the samples (Figure 1). Afterward, a nested-PCR test was employed to assess the HCMV DNA. According to the results, among 50 cases of breast IDC, viral 174-bp fragments had proliferated in 26 (56%) cases, i.e. they were positive for HCMV DNA. A total of 17 (65.4%) and nine (34.6%) cases of the breast IDC tissue, respectively in the moderately differentiated and the poorly differentiated stages, were positive for HCMV DNA. In accordance with the statistical analysis, there was a statistically significant association between HCMV status and histological grade in breast carcinoma cases(p-values<0.05).

Table 1: Distribution of HCMV based on histology grade and age in patients with breast cancer.

Frequency of HCMV variables		Positive sample (%)	Negative sample (%)	Total	p-value
Age	45>	10 (38.5%)	14 (58.3%)	24	0.16
	≥45	16 (61.5%)	10 (41.7%)	26	
Histology grade	G1	0	5 (20.8%)	5	0.04
	G2	17 (65.4%)	12 (50%)	29	
	G3	9 (34.6%)	7 (29.2%)	16	

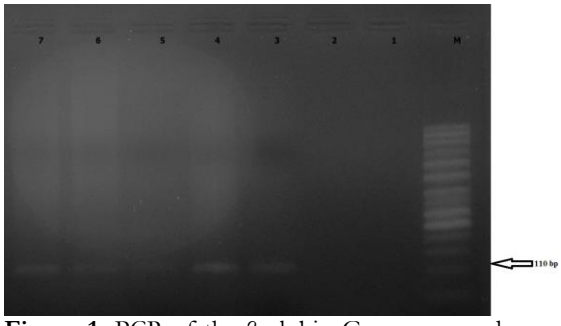

Figure 1: PCR of the β-globin Gene was used as an Internal Control for DNA Extraction. Lanes 3-7: PCR Products of β-Globin Gene (110bp Fragment); M: 50 base Pairs DNA Ladder; lanes 1-2: Negative Control.

Statistical analysis

The results were analyzed using SPSS version22.0. Chi-square and Fisher's exact tests were performed in order to have statistical comparisons. Corresponding tables have been produced to discuss the results. P-values< 0.05 were considered statistically significant.

Discussion

HCMV is a human beta-herpes virus that the host's immune system is unable to eliminate following initial infection. The virus remains dormant in the body and can be reactivated at any time during the person's lifetime (24).Breast milk is one of the most frequent routes of transmission of the virus, and breast epithelium might be a major site of latent and active HCMV infection. Since cell free virus is shed in the breast milk in virtually all HCMV seropositive females, breast glandular epithelial cells are likely the natural reservoir for HCMV in the breast(11).

Late exposure (in adulthood) to a particular virus, such as human cytomegalovirus, has been hypothesized as a risk factor for breast carcinomas. It is suggested HCMV could be associated with breast cancer because it is a ubiquitous virus that is shed in breast milk, as well as in saliva, urine, cervical secretions, and semen, implying HCMV persistently infects epithelial cells (25). The virus employs strategies to escape the innate and acquired immune system so as to establish dormant and stable infection in the body. In cancer patients, the virus comes out of the latent phase and exhibits oncogenic characteristics(24). Reactivation of HCMV in patients with cancer can be influenced by polymorphisms of cytokine genes. These findings support a relationship between HCMV and carcinogenesis(26).In this study, we investigated the frequency of HCMV in tissue samples of 50 women suffering from breast cancer by using nested-PCR. Several studies indicated that HCMV infection has been linked to the malignant phenotype, particularly with glioblastoma (4).Many studies have indicated a association between HCMV with breast cancer, but they have shown different results(19). Dolores Utrera-Barillas1 and his colleagues were unable to support the hypothesis that HCMV infection might be correlated with tumor progression. The only probable mechanism that could rationalize this finding is the "hit-and-run" hypothesis, which claims that viruses could mediate cellular transformation through an initial "hit", whereas maintenance of the transformed state is considered as the result of viral molecule ("run") loss. Although the concept of hit-and-run transformation is still on debate, it remains the only logical explanation for the observations of neoplastic transformation following in vitro transfection of herpesvirus and cytomegalovirus DNA(4). By using serological methods, AK Richardson and his colleagues indicated that late exposure to CMV could be considered as a risk factor for breast cancer development. However, an association between breast cancer and CMV might occur if CMV infection serves as a surrogate of a similar viral infection (25).

late exposure to CMV in adults could Plays an important role in some virally infected cell types to generate one or more genetic Mutations which required for the development of breast cancer. The cases in which HCMV-infected cells are existed in the tumor microenvironment, the secretion of CMV IL-10 could probably triggers the development of a more invasive tumor phenotype. Cancer cells that replicate faster and express fewer adhesion molecules are more likely to dissociate from the primary tumor. These events show a probable role for cmvIL-10 in promoting malignancy and also suggest that HCMV-positive cancer patients could be targeted for the anti-viral therapy, enhancing their survival time (27). Mohamed El-Shinawi and his colleagues were shown a statistically significant difference in HCMV-DNA detected in carcinoma tissue of IBC compared with versus non-IBC patients (7). This study indicated a significant result of the presence of HCMV DNA in the invasive ductal breast carcinoma tissue, regardless the grade of differentiation. This result was confirmed by Harkins LE *et al.*, 2010 when they used PCR and in situ hybridization to detect HCMV DNA in the breast ductal carcinoma in situ (DCIC) and invasive ductal carcinoma (IDC) (4) and viral

DNA was observed in 26 (58%) out of the 50 cancerous tissue samples. We were able to support the finding that HCMV infection may be correlated with breast carcinoma.

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