RESEARCH ARTICLE

Detection of Human Cytomegalovirus in patients with Colorectal Cancer by Nested-PCR

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Abstract

Background: The association of colorectal cancer with human cytomegalovirus (HCMV) is a controversial issue in cancer research. This study aimed to identify the HCMV virus in colorectal cancer tissues and to investigate the association of HCMV with colorectal cancer. In this study, 50 cancer tissue samples and 50 samples without colon cancer were studied in order to identify the HCMV virus through nested-polymerase chain reaction. The virus was identified in 15 cases of colorectal cancer tissues (15/50) and in 5 cases of normal tissues (5/50). Eight cases of adenocarcinoma tissues were in a moderately differentiated stage, and 7 cases had well-differentiated stage tissues that were positive for viral DNA. The findings were statistically evaluated at a significance level of $p<0.05$. The HCMV virus could play a role in creating malignancy and the progress of cancer through the process of oncomodulation.

Keywords: Colorectal cancer - human cytomegalovirus - nested-PCR

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Introduction

Colorectal cancer is the third leading cause of cancer-related deaths worldwide, with nearly one million new cases identified annually. In recent years, several risk factors associated with carcinogenesis have been proposed, including; environmental factors, life style, genetic changes, and viral infections (Perkin et al., 2002). Viral etiology in cancerous malignancies is a very important issue and so far, a number of viral strains have been identified as tumor oncogene viruses, including; HTLV-1, EBV, HCV, HBV, HPV, and herpes viruses (Fearon et al., 1990; Enam et al., 2002; Damin et al., 2007).

The human cytomegalovirus (HCMV) is a type of β-herpesvirinae, a sub-family of Hepadnaviridae. HCMV prevalence among different populations worldwide varies from 45% to 100%, according to geographical location and socioeconomic level (Dowd et al., 2009). The HCMV seroprevalence is more prevalent in Asia and Africa than in Western Europe and the United States (Cannon et al., 2010).

HCMV is considered to be one of the common causes of gastrointestinal diseases, particularly in immune-compromised patients. A HCMV infection is able to infect all parts of the digestive tract, from the mouth to the rectum, causing mucosal ulcers and it is often accompanied with bleeding. Following infection with this virus, HCMV remains dormant for a long time. The HCMV genome and its associated proteins have been observed in neoplastic cells, but they have not been seen in the adjacent, normal tissues. It is believed that malignancies caused following an HCMV infection, are the result of reactivation of the dormant virus in the body, and that causes numerous problems especially in immune-compromised people, and those receiving immunosuppressive medication (Harkins et al., 2002). HCMV proteins and nucleic acids are detected in some tissue specimens including mucoepidermoid salivary gland tumours (Melnick et al., 2012), glioblastoma (Rahbar et al., 2013), neuroblastoma (Wolmer-Solberg et al., 2013), rhabdomyosarcoma (Price et al., 2012). Studies have shown that virus cloned DNA fragments have the ability to transform rodents' cultured fibroblast cells. According to these observations, the theory of 'hit and run,' has been proposed in relation to viral function in the colon cells (Michaelis et al., 2009). According to this theory, it appears that HCMV first begin to proliferate in the cells and then it causes cell transformation, followed by induced cell carcinoma (Shen et al., 1997; Bender et al., 2009; Chiba et al., 2013). The association of HCMV with colorectal adenocarcinoma was first reported in 1978 by Huang and Roche (Huang et al., 1978), and ever since, various studies in different societies have confirmed the association between this virus and colorectal cancer. However, the details of this association still remain unclear, as there are conflicting reports which could be due to the methodology used and/or the number of populations studied.

The present study aims to identify the virus in patients with colorectal cancer through the accurate nested-PCR method. Therefore, if a significant association is found,

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appropriate treatment methods can be used to prevent the cancer from progressing.

Materials and Methods

Study group

All specimens were provided by the Pathology Department of Imam khomeini Hospital (Tehran-Iran). Specimen were investigated by two pathologists then used for DNA extraction and PCR analysis. The specimens included in the study consisted of 50 Formalin-fixed tissue of colorectal cancer including 8 colorectal adenomas and 42 colorectal adenocarcinomas and 50 normal tissues with no malignancy. Of 42 adenocarcinomas, 10 were well differentiated, 30 moderated differentiated and 2 poorly differentiated.

Genomic DNA extraction

Total cellular DNA was extracted from samples by General Genomic Extraction Kit according to the manufacturer's instructions. This kit was newly developed by the first author of the paper. Briefly, 0.05g of chopped tissues were mixed with lysis buffer (Solution A), and 30µl proteinase K (20mg/ml) in 1.5ml microtubes and incubated at 65˚C for 3h. Microtubes were inverted each 15minutes for good solution of the crushed tissues with the buffer. 600µl of Binding Buffer (solution B) was added and centrifuged at 12,000rpm for 5min. Upper aqueous phase was separated without disturbing the interphase. This step was repeated once again. The aqueous phase in each tube was transferred to a new1.5ml microcentrifuge tube. 600µl of cold Precipitation Buffer (Solution C) was added and inverted for 20min. The resultant mixture was centrifuged at 12,000rpm for 10min and the upper aqueous phase was removed. The DNA pellet was washed with cold Washing buffer (solution D) followed by 15min mild inversion at room temperature and centrifugation at 12,000rpm at 4˚C for 10min. Washed DNA pellet was dried by leaving the tubes in a 37°C for 40minutes. DNA sample was dissolved in 50µl Solvent Buffer. Genomic DNA purity was assessed with a NanoDrop™ ND-2000 spectrophotometry and calculated by ratio of the DNA optical density (OD 260) and protein optical density (OD 280). Genomic DNA yield was calculated from DNA optical density (OD 260) for clean DNA samples.

Verification of DNA extraction

All samples were examined for DNA integrity using amplification of the β-globin. Sequences of primers were: PC04: 5' CAA CTT CAT CCA CGT TCA CC 3', GH20: 5' GAA GAG CCA AGG ACA GGT AC 3'. PCR were carried out in a final volume of 25µl containing 12.5µl of Amplicon master mix, 0.5µl of forward and reverse primers and 1µl of DNA template. Amplification was carried out in Thermal Cycler (Biorad). After an initial denaturation step at 95°C for 3min, 45 cycles were programmed as follows: denaturation step at 95°C for 30 sec annealing step at 53°C for 40 sec, primer extension at 72°C for 40 sec and final extension step at 72°C for 5min. PCR products were determined by visualization of amplicons on 2% agarose gels stained with gel red.

Nested PCR amplification

The first-round PCR amplification was performed in a 25µL reaction volume containing 12.5µl of Amplicon master mix, 0.5µl of forward and reverse primers, 10µl of each genomic DNA sample. Sequences of oligonucleotides primers were: 5'TCCAACACCCACAGTACCCGT-3' and 5'CGGAAACGATGGTGTAGTTCG-3' (Kühn et al., 1995). PCR program was performed as follows: pre-denaturation at 95°C for 5minutes, 1 cycle; denaturation at 94°C for 30 seconds, annealing at 55°C to for 30 seconds, extention at 72°C for 30 seconds, 20 cycles; post- extention at 72°C for 5minutes, 1 cycle.

The second-round PCR amplification was carried out by using 12.5µl of Amplicon master mix, 0.5µl of forward and reverse primers and 5µl of the first round PCR products as templates in 25µL reactions. The sequences of the inside primer pair were: 5'-GCCCGCCGCGGCAGCACCTGGCT-3' and 5'-GTAAACCACATCACCCGTGGA-3' (Kühn et al., 1995). PCR cycling protocol was used: 95°C for 5 minutes, 1 cycle; 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, 30 cycles, 72°C for 5minutes, 1 cycle.

At the end of amplification, 5µL of the PCR products was analyzed on 2% agarose gel. The resultant product was expected to be a 174-bp fragment.

Statistical analysis

Statistical analyses were performed using the SPSS (version 14.1) software package. Frequency tables were analyzed using the Chi-square test, with Fischer's exact test. $p< 0.05$ was accepted as statistically significant.

Results

The mean age of the subjects was 52 years (youngest 16 and oldest 79 years-of-age). The tissues studied included 42 colorectal adenocarcinoma and 8 colorectal adenoma tissues. The adenocarcinoma tissues were categorized in one of three stages; poorly, moderately, and well differentiated. To extract DNA from the tissues, β-globin primer was used to conduct a PCR. After verification of the 268-bp fragment, samples were selected for the nested-PCR. Given the quality and quantity of extracted DNA, 268-bp fragments proliferated in all samples (including normal and cancerous tissues) (Figure 1). Then, a nested-PCR test was used to evaluate the viral DNA.

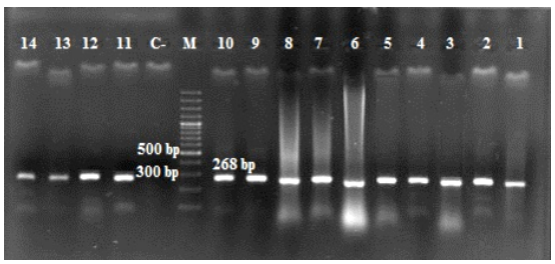

Figure 1. PCR of the β-globin Gene was used as an Internal Control for DNA Extraction. Lanes 1-14: PCR Products of β-Globin Gene (268bp Feragment); M: 100 base Pairs DNA Ladder; C-: Negative Control.

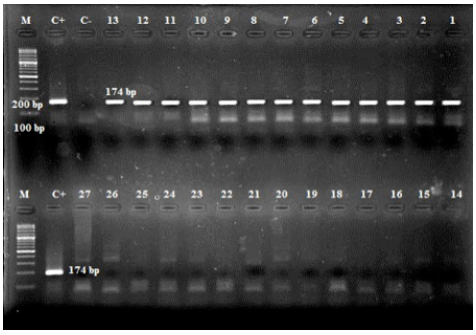

Figure 2. Profile of Nested-PCR for Detection of HCMV Gene (174-bp PCR product) on 2% Agarose Gel. Lanes 1-13: Detection of HCMV in colorectal cancer tissues; c-: Negative control; c+: Positive control; M: 100 base pairs DNA Ladder; Lanes 14-27: some normal tissues.

Results showed that viral 174-bp fragments proliferated in 15 cases of colorectal cancer tissues (15/50) and 5 cases of normal tissues (5/50) (Figure 2). Eight cases of adenocarcinoma tissues in the moderately differentiated stage and 7 cases in the well-differentiated stage were positive for viral DNA. The results were analyzed using SPSS (version 14.1) software with a significance level of $p<0.05$ ($p=0.011$).

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. The virus employs strategies to escape the innate and acquired immune system in order to establish dormant and stable infection in the body. In cancer patients, the virus comes out of the latent phase and exhibits oncogenic characteristics (Derberg-Naucle, 2006). Reactivation of HCMV in patients with cancer can be influenced by polymorphisms of cytokine genes. These findings support a relationship between HCMV and carcinogenesis (Cano et al., 2012).

In vitro studies have shown that HCMV is able to transform cells, and cause disruption along many cell paths, which can eventually lead to pathogenicity and colon adenocarcinoma (Al-Sohaily et al., 2012), in addition it is also able to effect; the cell cycle, mutagenesis, apoptosis, angiogenesis and oncogenesis (Michaelis et al., 2009; Shen 2011). In the 1970's, the Fred Rapp research group reported that HCMV is able to transform normal cells in vivo (Geder et al., 1976). Moreover, the presence of HCMV genome and antigens have been reported in malignant tumors such as; colon cancer (Harkins et al., 2002), malignant gliomas (Cobbs et al., 2002), Hodgkin's lymphoma (Huang et al., 2002), breast cancer (Harkins et al., 2010), and prostate cancer (Samantha et al., 2003), while the non-cancerous cells adjacent to tumor cells were negative for HCMV.

In the present study, the presence of HCMV DNA in colorectal adenocarcinoma tissues was confirmed using the nested-PCR method, and viral DNA was observed in 15 (30%) out of the 50 cancerous tissue samples. These results were unlike those found by other researchers, who found an insignificant relationship between HCMV virus and colorectal cancer (Ruger et al., 1985; Knosel et al., 2004; Mariguela et al., 2008; Bender et al., 2009). In this present study, the results were similar to other studies on colorectal cancer, which point to a relationship between viral infection and the incidence of colorectal cancer (Huang et al., 1978; Harkins et al., 2002; Akintola-Ogunremi et al., 2005; Chen et al., 2012). Unlike Bender's results indicating the absence of HCMV in advanced adenocarcinoma samples, in this study the presence of HCMV in 7 samples out of 15 advanced adenocarcinoma tissue were confirmed through a nested-PCR procedure. Moreover, no HCMV was observed in any of the adenoma samples. Recently, preferential infection of neoplastic epithelium cells by HCMV in colorectal cancer has been cited (Chen et al., 2012). Recently, high prevalence of human cytomegalovirus in brain metastases of patients with primary breast and colorectal cancer has been detected (Taher et al., 2013). Dimberg's observations declares that the prevalence of HCMV DNA is significantly higher in cancerous in relation to normal tissue (Dimberg et al., 2013).

Various conflicting results among these studies could be the result of a range of factors that impact on the results, including; study limitations and methodology, as well as the study population used to detect the virus. It is recommended that more powerful techniques and larger statistical populations be used in future studies. Histochemical methods were used in a number of studies. In this method, viral proteins that are produced in acute infections are identified (Knosel et al., 2004). Since commercial and specific antibodies of wild-type proteins are present in the histochemical studies, reports of negative results are possible, and this is because it is possible that viral mutant-type proteins are expressed in the neoplastic epithelium cells of colorectal cancer (Chen et al., 2012). PCR is able to identify both acute and dormant infections. Furthermore, the type of tissue studied is also important because it is possible that identifying viral DNA in tissues kept in formalin for a long time will fail when compared to fresh tissues (Rogers et al., 1990; Koshiba et al., 1993). In studies in which the PCR method could not identify viral DNA, because of the 'hit and run' theory in tumorigenesis and its progression, researchers did not totally reject non-viral infection as a factor (Bender et al., 2009; Akintola et al., 2005). According to this theory, the virus infects the cell in the early stages of the infection, then transforms the cell and induces mutation in the cell genome. In addition, the HCMV virus oncomodulatory mechanisms induces changes in the cell biology including; preventing apoptosis in cancerous cells, cell cycle progress, stimulating angiogenesis (Hanahan and Weinberg, 2011), disrupting intracellular signalling pathway, and increasing telomerase activity in these cells, all of which cause tumor growth and more importantly, resistance against chemotherapy (Cinatl et al., 2004). These changes are not observed in normal cells.

The HCMV remains a serious pathogen in immune-compromised patients. HCMV gene products may also alter the apoptotic potential through activation of

PI3K/AKT and Wnt/β-catenin signalling pathways. By activating the signal pathway associated with survival and growth of cells, HCMV may increase and reinforce cancerous cells' malignant properties (Bongers et al., 2010). It may also help the survival of cancerous cells through regulation and modulation of the immune system. Furthermore, if tumor cells have been infected with HCMV for a long time, it seems that viral proteins prevent the immune system from their function to identify and eliminate these cells and consequently, this will lead to the progression of cancer and tumor (Cinatl et al., 2005). This status should be considered in the development of cancer immunotherapy methods, particularly because HCMV infected cells may resist against the function of cytolytic peptides released by natural killer cells and activated T-lymphocytes (T-cells). Additionally, if apoptosis inhibiting viral proteins are expressed then cancerous cell sensitivity to the commonly used chemotherapy methods will be reduced. By confirming the role of HCMV in colorectal cancer pathogenesis, the important role of antivirals and cancer progress prevention can be addressed by preventing the proliferation of the virus through antiviral medication (Cinatl et al., 1998).

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