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DETECTION OF HUMAN CYTOMEGALOVIRUS IN IRAQI COLORECTAL CANCER PATIENTS

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ABSTRACT

Background: Human cytomegalovirus (HCMV) is a beta-herpesvirus that causes persistent infection in humans and can cause severe disease in fetuses and immune compromised individuals. Although, it was hypothesized that HCMV might be associated with colorectal cancer progression. However, the role of HCMV infection in colorectal cancer remains controversial.

Objectives: Detection of cytomegaloviral DNA in cells of colorectal cancer among Iraqi patients by polymerase chain reaction (PCR).

Patients and methods: Sixty patients who had colorectal cancer as confirmed by histopathological examination of tissue specimens from the affected lesion. The patient's ages were ranged between 21-81 years, 12 (34.3%) were females and 23(65.7%) were males. Additionally, fifty apparently healthy individuals who had no history of malignant diseases were enrolled as a control group. 22 (44%) were females and 28 (56%) were males with age range between 20-70 years. Detection of anti HCMV IgG and IgM using the Bio ELISA Kits (Biokit S.A. Spain). Cancerous tissue biopsies were obtained during endoscopy and were submitted for DNA extraction and processed for PCR technique. Human privacy was respected by taking subjects consent as part of study design.

Results: The results showed that 35(58.3 %) out of 60 patients were positive for anti- HCMV IgG, while all of them were negative for anti-HCMV IgM. All the 35 (100%) patients who were positive for anti-CMV IgG were also positive for CMV genomic DNA, as demonstrated by the presence of specific bands.

Conclusion: These findings point out to the role of HCMV in the development and or progression of colorectal cancer among Iraqi patients.

INTRODUCTION

Human cytomegalovirus (HCMV) or human herpesvirus-5 (HHV-5) is a member in the subfamily *Betaherpesvirinae* of the family *Herpesviridae*. HCMV is ubiquitous, latently infects between 50% and 80% of adults worldwide, reactivated virus can cause life-threatening disease in immune compromised patients (Reeves and Sinclair, 2008). Gastrointestinal tract infection due to HCMV can occur in all location from mouth to rectum. A possible association between HCMV infection and colorectal cancer progression has been inferred by the identification in tumor tissues of HCMV antigens and specific viral DNA or RNA sequences (Harkins *et al*., 2002; Collins *et al*., 2011). On the other hand, several observations suggest that there is no evidence of a direct association between HCMV and colorectal cancer (Bender *et al*., 2009; Akintola-Ogunremi *et al*., 2005). So far, the causative role for HCMV in the development of the neoplasias remains to be established. However, this puzzling dilemma has been better explained by the concept of "Oncomodulation" which means that HCMV infects tumor cells and increases their malignancy as proposed by molecular mechanisms and clinical findings (Michaelis *et al*., 2009; Michaelis *et al*., 2011). Additionally, *in vivo* study on human malignant glioblastoma cells found that the HCMV immediate early-1 (IE1) protein that detected in >90% of these tumors is essential for viral infection and has potent trans-activating and oncomodulatory properties. (Cobbs *et al*., 2008). Likewise, it has been suggested that in persistently HCMV-infected tumor

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cell lines - a selection of novel, slowly growing virus variants with changes in coding sequences for virus regulatory proteins takes place resulting in oncomodulatory effects that may lead to a shift to more malignant phenotype of tumor cells contributing to tumor progression (Cinatl *et al*., 2004).

METERIALS AND METHODS

Study groups

Sixty inpatients from those attended the Gastroenterology and Hepatology Department in the Medical City Teaching Hospital-Baghdad, and AL-Mustansiriyah Private Hospital-Baghdad during the period from October 2009 to November 2010, were enrolled in this study. They were all initially suspected as having colorectal cancer on the clinical ground. The confirmatory diagnosis was achieved by ultrasonography, sigmoidoscopy, and histopathological examination of tissue specimens from the affected lesion. The patient's ages were ranged between 21-81 years (average of 52.4 years), 12 (34.3% were females and 23(65.7%) were males. A special questionnaire was pre-constructed and filled for each patient by short interview. Demographic and medical information were collected, including age, sex, socioeconomic status, and family history of CRC, medical history of the disease, disease manifestation, and co-existence of other disease. Patients with previous chemotherapy, radiotherapy, or corticosteroid therapy were excluded from the study. Additionally, fifty apparently healthy individuals were enrolled as a control group. They consist of 22 (44%) of females and 28 (56%) of males with age range between 20-70 years. All had no history or clinical evidence of malignant diseases. Human privacy was respected by taking subjects consent as part of study design.

Collection of samples

A-Blood samples

Ten milliliters of whole blood were collected from each of 60 patients. Each blood sample was divided into two parts; 5 milliliters were placed into EDTA anti-coagulated tubes and kept frozen at -20 ^{0}C until use, for DNA extraction and PCR technique. Another 5 milliliters was placed in disposable plane tubes for separation of serum. Separated sera were subdivided into many liquate in Eppendorf tubes (250 micro liter tubes), kept frozen at -20 c˚ until use. The 60 serum samples were subjected to enzyme linked immunosorbent assay (ELISA) for the detection of Anti HCMV IgG and IgM using the Bio ELISA Kits (Biokit S.A. Spain) IgG and IgM anti HCMV, following manufacturer's instructions.

B-Tissue specimens

The tissue specimen included in this study consisted of 10 milligrams of tumor tissue were obtained from only 35 out of 60 patients during endoscopic examination or during operation. These 35 patients were positive for anti HCMV IgG by ELISA. Tissue specimens were embedded in paraffin blocks and were kept at room temperature until use.

DNA extraction from blood samples and CRC tissue biopsies

Blood and tissue biopsy samples that showed positive results for anti HCMV in ELISA test were used for DNA extraction. For separation of peripheral blood leukocytes (PBL), 5 milliliters of EDTA, treated whole blood samples were collected. Briefly, 1 milliliter of 1% dextran was added to each blood sample and the mixture was incubated at 37 ^{0}C for 30 minutes. Four sections of 3 micrometer thickness of each paraffin embedded CRC tissue sample were deparaffinized with xylol/ethanol and collected into a 1.5 milliliter eppendorf tubes containing 100 microliters of lyses solution. The treated tissues were stored at -20^{0}Cuntil use. DNA extraction from above-mentioned samples was performed as described by Sambrook *et al*. (1989). Furthermore, the concentration and purity of extracted DNA was determined as described by Rodriguez and Tait (1983).

PCR (DNA amplification)

PCR technique is performed at Forensic Medicine Institute, Baghdad and AL-Karama Hospital, Baghdad. DNA PCR Bio kit (DYNAL, USA) was used to detect HCMV in samples of the present study. PCR was carried out in 50 microliter total volume. The reaction mixture contained 10 micromole Tris hydrochloride (pH8.3), 50 micromoles KCl, 1.5 millimole $MgCl_2$, 100 micrograms of gelatin per ml , 300 micromoles each of deoxynucleotides, 1.0 micromole of each oligonucliotide primer (A and B).

A (5´-TCCAACACCCACAGTACCCGT-3´)
B (5´GGAAACGATGGTGTAGTTCG-3´)

1.255 units of thermos Aquaticus DNA polymerase (Ampli Taq), DNA Bio kit, (DYNAL, USA) and 10 microliters of the target DNA solution. This mixture was overlaid with 50 microliters of light mineral oil. First denaturation for 1 minute was at 94˚C, annealing for 1minute 30 seconds at 63^{0}C and extension for 1 minute at 72 ^{0}C. Thermal cycling was done in programmable (30 cycles) thermal controller. To avoid contamination, no positive control was used for PCR at the time as clinical samples, and great care was taken to extract all DNA samples in separate room from where PCR reaction was carried out. Positive controls were included in each run from PCR Bio kit to ensure that no DNA contamination occurred. To optimize detection of HCMV, primers were evaluated and PCR amplification condition for HCMV genes with primers sets in their capacity to amplify limited dilution of an HCMV genomic control template.

Analysis of DNA (Gel electrophoresis)

Ten microliters of amplified DNA were mixed with 1 microliter of loading buffer. The mixture was loaded in 2% agarose that containing 0.5 microgram per ml ethidium bromide run in TBE buffer (0.045 mole Tris borate, 0.0001 mole EDTA, pH 8.0). The electrophoresis was carried out at 50 volts for 1 hour, by the use of BioRad Model 200/2.0 power supply. All PCR tested CRC samples were flanked by positive control amplified HCMV Gp gene and molecular weight marker as provided by the manufacturer (DYNAL, USA). DNA bands were then detected using a UV transilluminator and photographed by photo document system.

Results

Description of study groups

The patients group included 60 patients with CRC, 35 of them that have positive test result for Anti-CMV IgG. Their ages

ranged between 21-81 years. 12 (34.3%) of them were females and 23 (65.7%) were males, all with mean age 52.4 ± 16.3 years. Fifty apparently healthy individuals were age and sex matched enrolled as control group. Their ages ranged between 20-70 years. 22(44%) were females and 28 (56%) were males, all with mean age 45 ±17.2 years, table (1). Forty two percent (42%) of the patients were below 40 years, and 58% of CRC patients were above 40 years, figure (1).

Table 1. Age and sex distribution among study groups

Age in year	CRC patients	control group
Minimum	21	20
Maximum	81	70
Mean ± SD	52 ± 16.3years	45 ± 17years
Total	35	50
Gender		
Male	23	28
Female	12	22
Total	35	50

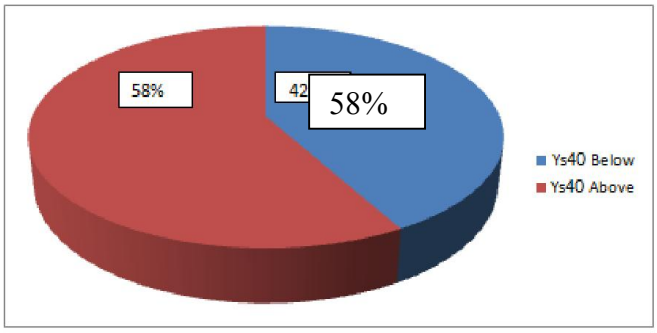

Figure 1. Distribution of colorectal cancer patients according to the age

Determination of anti- CMV IgG and IgM

Sixty patients were checked for HCMV infection in their sera 35(58.3 %) of them were positive for anti- HCMV IgG and all of them were negative for anti-HCMV IgM. For the healthy control, 15(30%) sera were positive for the presence of anti-CMV IgG, figure (2).

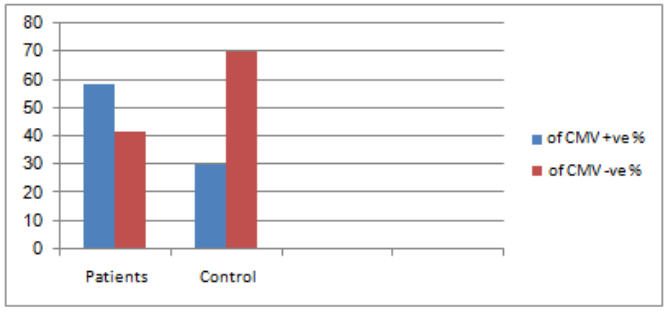

Figure 2. Description of study groups according to CMV infection

Detection of HCMV genome by PCR

Anti-HCMV, IgG positive sera were subjected to PCR techniques by the use of whole blood samples of selected CRC patients. The results showed that 35 (100%) of them were positive for CMV genomic DNA, as demonstrated by the presence of expected bands (104 bp), (Figure 3). To ensure adequate quality of genomic DNA prepared from tissue blocks, PCR analysis for the presence of HCMV genomic DNA was performed. The results showed that 20 (57.1%) samples out of 35 were positive for HCMV genomic DNA when amplified effectively to a very similar extent under the same PCR amplification conditions used for CMV with expected band (104 bp), Figure (4).

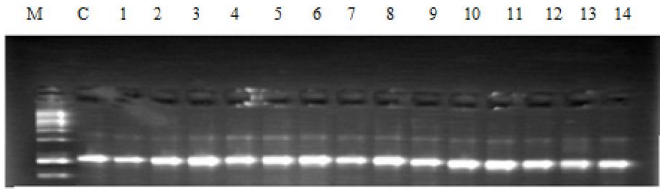

Figure 3. CMV PCR performed on CRC blood samples. Lane M is the molecular weight marker. Lane C is the positive kit control Lanes, 1 to 14 are some of CMV PCR positive samples

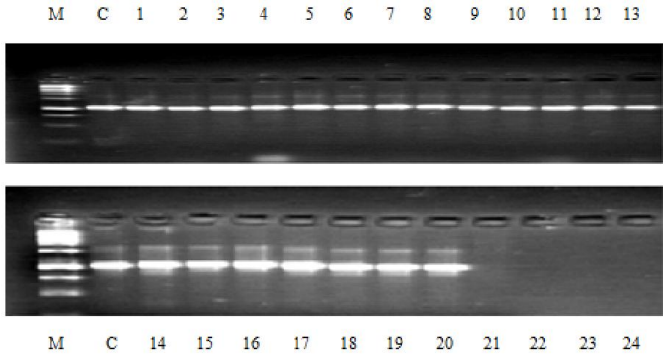

Figure 4. CMV PCR performed on CRC tissue samples. Lane M is the molecular weight marker. Lanes C is the positive kit control. Lanes, 1 to 20 are CMV PCR positive samples. Lanes, 21, 22, 23, and 24 are some of CMV PCR negative samples

DISCUSSION

The importance of the present study may be arise from several points; the high prevalence of anti-HCMV antibody among Iraqi cancer patients that may reach up to 100% (Hasan and Omer, 2005), the increased incidence of colorectal cancer particularly during the last few years (Iraqi Cancer Registry Results, 2005), and for the best of our knowledge there was no previous study to assess the correlation between HCMV infection and colorectal cancer in Iraq. The present study showed wide range of age incidence of CRC between 21-81 years old with only one case was observed with Familial adenomatous polyposis, the age of incidence was 42% below 40 years and 58% was above 40 years. This observation was not in agreement with previous studies showed that the most cases of CRC occur in 60s and 70s, while cases before age 50 years were uncommon unless a family history of early colon cancer is present (Winawer *et al*., 1984; Du *et al*., 2006). This finding may be attributed to the change in pattern of CRC in Iraqi population, probably due to the rapid change in dietary habits, beside the possible effects of environmental and chemical hazard of wars during the last decades (AL-Bahraini and AL-Humadi, 2008). This study showed a higher rate of CRC among males (65%) versus females (34%). So, the present study is consistent with other studies that showed that men were in the highest quintile for body size classifiable as obese, had as much as twofold increased risk of colon cancer with little such association in women, beside the high alcohol consumption and cigarette smoke among men (Potter *et al*., 1996; Wier *et al*., 2003). The serological analysis showed high

frequency of anti-CMV-IgG among CRC patients compared to healthy controls. It is well documented that immune-compromised patients including cancer patients had high frequency of anti- CMV IgG (Bender *et al*., 2009; Hasan and Omer, 2005; Mariguela *et al*., 2008). The present study also showed that 15 (35%) individuals are positive for anti-HCMV IgG, and this may be attributed to past infection, since in our community the majority of infection by HCMV occurs during the childhood (Abdul-Karim *et al*., 1989). The absolute negativity of anti- CMV IgM among subjects in the present study may be due to the fact that reactivation of CMV and the reappearance of IgM specific antibody occur in the terminal stage of immunocompromisation, when the peripheral CD_4 count dropdown to 200 cell/milliliter (Pawelec *et al*., 2009). The presence of CMV DNA genome in all blood samples that matched antibodies against CMV by ELISA technique may be attributed to the fact that leukocytes are among the sites of CMV latency (Mazeron, 2000). On the other hand, HCMV genome was detected in 20 paraffin embedded tissue biopsies out of 35 (57.1%) of CRC patients.

This result led to the suggestion that the presence of CMV in tissue biopsies may be associated with CRC progression. This suggestion may be attributed to the presence of viral protein that can block the induction of apoptosis by the release of tumor necrosis factor-alpha or viral IE protein stimulate cell cycle progression by inducing protein degradation of another important tumor suppressor genes and it's family members P170 and P130 (Michaelis *et al*., 2011 ; Cobbs *et al*., 2008 ; Zhu *et al*., 1995). This result came in agreement with the findings of Mariguela *et al*., (2008), that 64.3% of cases of CRC were positive for the presence of HCMV DNA in tissue biopsies. The same authors found CMV DNA in only 21% of CRC patients when they use nested PCR technique. Furthermore, Bender *et al*., (2009), found that only 11% of cases were positive for the presence of HCMV DNA in paraffin embedded tissue biopsies, when they used advanced technique of PCR. The results of present study were nearly of the same findings reported in cases of malignant glioma caused by HCMV (Cobbs *et al*., 2008; Cobbs *et al*., 2002) that found HCMV DNA genome in 71.4% of tissue biopsies. In a final conclusion, HCMV DNA was detected in about one half of tissue biopsies of Iraqi CRC patients. These findings actually point out to the questionable role of HCMV in development of CRC among Iraqi patients. However, these results are waiting to be obeying or denying by further studies.

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