Detection of Epstein-Barr virus and Human Cytomegalovirus in Colorectal Cancer Tissue by Real Time PCR

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Abstract

Background: Epstein–Barr virus (EBV) and Human Cytomegalovirus (HCMV) are ubiquitous viruses. Many researchers studied the association of these two viruses with colorectal adenocarcinoma. This study aimed to investigate the frequency and viral load of EBV and HCMV in colorectal cancer tissues.

Subjects and methods: A case-control study included 40 colorectal cancer (CRC) formalin-fixed paraffin-embedded (FFPE) tissue samples, and 20 normal colonic FFPE tissues. DNA was extracted from these tissues and subjected for real time PCR quantification of EBV and HCMV DNA copies in these tissues.

Results: Among these 40 CRC tissues, HCMV was detected in 20% (8/40) of CRC tissue and 5% (1/20) of normal tissues, p=0.064, with significantly higher frequency in rectal cancers (8/8) and in stages 3 and 4 tumors, while EBV was detected in 25% (10/40), and in 20% (4/20) of normal tissues, P=0.672, with no significant association with tumors’ site, grade or stage. Conclusion: Human CMV could be considered an important risk factor in the development of rectal cancers; however, EBV is not significantly associated with development of CRC.

Key words: EBV, HCMV, colorectal cancer, quantitative real time PCR

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List of abbreviations:
EBV: Epstein–Barr virus
HCMV: Human cytomegalovirus
CRC: Colorectal cancer
qPCR: quantitative real time PCR
FFPE: Formalin-fixed paraffin-embedded tissues

Introduction

Colorectal cancer (CRC) can be considered as the third most commonly diagnosed malignancy in men and the second most common tumor in women worldwide. The incidence of colorectal cancer differs
around the world (1). And due to its high prevalence and mortality rates, it is one of the main global cancer related dilemmas (2).

Colorectal cancer can be categorized as inflammatory, inherited, or sporadic, which represent the majority of CRCs (about 80%) and it is the target of huge epidemiological researches to investigate the possible causative agents for this tumor (2,3). The gold standard that determines the prognosis of CRC patients is the tumor-node-metastasis (TNM) AJCC classification, which is based on histological and anatomical criteria of the tumor (4). CRC is a multifactorial disease originated from different interactions between hereditary, (epi) genetic, lifestyle, and environmental causes (5). Microbial-epithelial synergy was suggested as a possible oncogenic triggering factor for CRC development (6). However, a direct causal link between various microbial agents [e.g. Escherichia Coli, Helicobacter pylori, JC virus, CMV, EBV, and HPV] and CRC has not been established till this day (6-8).

EBV is a member of the herpesvirus family, it infects more than 90% of the world’s adult population (9). EBV causes multiply lympho-proliferative and epithelial malignancies including B-cell lymphomas (Hodgkin lymphoma, Burkitt lymphoma), gastric and nasopharyngeal carcinomas (10-12). The possible role of EBV in colorectal carcinogenesis has been investigated, and many researchers were able to find EBV DNA in colorectal adenocarcinomas and other cancers by various techniques, like in situ hybridization and PCR (13-15).

Human cytomegalovirus (HCMV) is a ubiquitous opportunistic herpesvirus that infects 70-100% of adults. HCMV remains a long lasting disease that can be reactivated at any moment causing in the long run high morbidity and even death particularly in immunocompromised patients (16-19). In the last two decades, the onco-modulatory capability of HCMV has been observed from studies with tumor cell lines infected by HCMV (20-21). There is an increasing number of recent data have demonstrated that HCMV is found within the colorectal tumor tissue (22-31).

There is a steady increase of CRC overtime and the underlying etiology is not known (32). Thus the present study was undertaken to find out the association of both EBV and CMV with CRC by quantitative real-time PCR assay and to clarify the clinical pathological features of these cancers.

Materials and Methods

This case-control study involved 40 formalin-fixed paraffin-embedded (FFPE) CRC tissue samples collected from (Gastroenterology and hepatology teaching hospital and Al-Imamin al-Kadhmain Medical City). Tissue samples were taken from endoscopic or surgical resection of tumors which were confirmed CRC in the period from March 2017 to September 2019. This study is approved by the IRB (institutional review board) of the College of Medicine-Al-Nahrain University (approval no. 20191022 on 13-11-2019). CRC were classified according to AJCC system (4). Also twenty FFPE histopathologically-proven normal colorectal biopsy tissues were studied as controls.

DNA Extraction: Tissue sections were taken from these FFPE blocks (both CRC and normal tissues), and put in xylene for 10 minutes then washed in 99% ethanol twice, and then, dried at room temperature. After that, the tissue sections were lysed by digestion buffer and then Proteinase K followed by centrifugation at 14000rpm for 10 minutes and the supernatant was taken for DNA extraction by using the QIA amp DNA mini kit (QIAGEN/Hilden/Germany) according to manufacturer instructions.

Quantitative real time PCR

Taqman probe real time PCR method used to quantify CMV and EBV DNA copies in colorectal tissue sections using (CMV Real-TM Quant) and (EBV Real-TM Quant) (Sacace-Italy) respectively, target genome for detection of CMV was polymerase gene and LMP gene for EBV. CMV-pol gene and EBV-LMP-gene DNA amplification were detected on JOE channel; and IC glob (β-globin gene) DNA amplification was detected on FAM channel. The real time-PCR instrument used was STRATAGENE MxPro (Agilent Technologies-USA). Thermal profile included initial activation of hot Start TaqP DNA Polymerase at 95°C for 15min; followed by 40 cycles of: denaturation at 95°C for 5sec, annealing at 60°C for 30sec, and extension at 72°C for 15sec, the fluorescence signal detected at the annealing step in each PCR cycle. The concentration of CMV DNA in 10^7 cells, calculated by the following formula:
The concentration of EBV DNA in $10^5$ cells, calculated by the following formula:

$$ \text{log} \left( \frac{\text{CMV DNA copies in PCR sample}}{\text{EBV DNA copies in PCR sample}} \right) = \log \left( \frac{\text{CMV DNA copies/10}^5 \text{ cells}}{\text{EBV DNA copies/10}^5 \text{ cells}} \right) \times 2 \times 10^5$$

where $10^5$ cells = $2 \times 10^5$ human genomic equivalents

**Statistical Analysis:** STATA statistical software version 14.2 was used. A logistic regression was used to estimate the odds ratios with 95% CIs by comparing the cases to the controls. The p value was calculated using a 2 sided Fisher's exact test. A p value less than 0.05 are considered significant.

**Results**

This case-control study involved 40 CRC tissues recruited from 16 (40%) males and 24 (60%) females, their mean age was 64.26±9.24. In addition to 20 control non-cancerous CRC tissues who were statistically comparable in age and sex. Real time QPCR analysis of HCMV in CRC tissues showed 20% (8 out of 40) positive, with a mean viral load $1.78 \times 10^6 \pm 1.7 \times 10^6$ copies CMV DNA/10$^5$ cells. And only one (5%) of the control tissues was HCMV positive, viral load was 3300 copies CMV DNA/10$^5$ cells. Out of the 8 HCMV positive CRC tissues seven were in the rectum ($P=0.003$), and all the 8 cases were among stages 2 and 3 ($p=0.022$), table (1).

Regarding EBV results, 40% (10/40) of CRC tissues were EBV positive, however, 20% (4/20) of the controls were also positive, $p=0.672$. In addition, there was no significant association between EBV positivity and pathological grading and staging system applied by AJCC, table (2). Only three patients (7.5%) had both HCMV and EBV in CRC tissues.
### Table (1): Descriptive and pathological criteria of HCMV results among patients and controls

<table>
<thead>
<tr>
<th>CMV</th>
<th>Negative</th>
<th>Positive</th>
<th>Total</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>Mean (years)</td>
<td>66.10±7.92</td>
<td>63.56±11.92</td>
<td>64.83±9.92</td>
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<tr>
<td></td>
<td>No.</td>
<td>51(85.0%)</td>
<td>9(15.0%)</td>
<td>60(100%)</td>
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<tr>
<td><strong>Gender</strong></td>
<td>Female</td>
<td>30 (58.8%)</td>
<td>6 (66.7%)</td>
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</tr>
<tr>
<td></td>
<td>Male</td>
<td>21 (41.2%)</td>
<td>3 (33.3%)</td>
<td>24</td>
</tr>
<tr>
<td><strong>Study groups</strong></td>
<td>Control</td>
<td>19 (37.3%)</td>
<td>1 (11.1%)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Patient</td>
<td>32 (62.7%)</td>
<td>8 (88.9%)</td>
<td>40</td>
</tr>
<tr>
<td><strong>Site</strong></td>
<td>Colon</td>
<td>29 (56.9%)</td>
<td>1 (11.1%)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Rectal</td>
<td>22 (43.1%)</td>
<td>8 (88.9%)</td>
<td>30</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td>Normal</td>
<td>19 (37.3%)</td>
<td>1 (11.1%)</td>
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</tr>
<tr>
<td></td>
<td>Poor</td>
<td>2 (3.9%)</td>
<td>1 (11.1%)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>25 (49.0%)</td>
<td>7 (77.8%)</td>
<td>32</td>
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<tr>
<td></td>
<td>Well</td>
<td>5 (9.8%)</td>
<td>0 (0.0%)</td>
<td>5</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td>0</td>
<td>19 (37.3%)</td>
<td>1 (11.1%)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10 (19.6%)</td>
<td>0 (0.0%)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>12 (23.5%)</td>
<td>4 (44.4%)</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10 (19.6%)</td>
<td>4 (44.4%)</td>
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</table>

### Table (2): Descriptive and pathological criteria of EBV results among patients and controls

<table>
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<th>Negative</th>
<th>Positive</th>
<th>Total</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>Mean (years)</td>
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<td>62.29±12.78</td>
<td>64.51±9.71</td>
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<td></td>
<td>No.</td>
<td>46(76.7%)</td>
<td>14(23.3%)</td>
<td>60(100%)</td>
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<tr>
<td><strong>Gender</strong></td>
<td>Female</td>
<td>26 (56.5%)</td>
<td>10 (71.4%)</td>
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<tr>
<td></td>
<td>Male</td>
<td>20 (43.5%)</td>
<td>4 (28.6%)</td>
<td>24</td>
</tr>
<tr>
<td><strong>Study groups</strong></td>
<td>Control</td>
<td>16 (34.8%)</td>
<td>4 (28.6%)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Patient</td>
<td>30 (65.2%)</td>
<td>10 (17.4%)</td>
<td>40</td>
</tr>
<tr>
<td><strong>Site</strong></td>
<td>Colon</td>
<td>22 (47.8%)</td>
<td>8 (57.1%)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Rectal</td>
<td>24 (52.2%)</td>
<td>6 (42.9%)</td>
<td>30</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
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<td>16 (34.8%)</td>
<td>4 (28.6%)</td>
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<tr>
<td></td>
<td>Poor</td>
<td>1 (2.2%)</td>
<td>2 (14.3%)</td>
<td>3</td>
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<tr>
<td></td>
<td>Moderate</td>
<td>24 (52.2%)</td>
<td>8 (57.1%)</td>
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<td>5 (10.9%)</td>
<td>0 (0.0%)</td>
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<tr>
<td><strong>Stage</strong></td>
<td>0</td>
<td>16 (34.8%)</td>
<td>4 (28.6%)</td>
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<td></td>
<td>1</td>
<td>8 (17.4%)</td>
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<td></td>
<td>3</td>
<td>10 (21.7%)</td>
<td>4 (28.6%)</td>
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</table>
Discussion

It is estimated that 16-18% of the global cancer burden can be associated with oncogenic viruses (33,34). Both HCMV and EBV are highly prevalent viruses in Iraq (35-37). The current study found border line significance in the difference between the frequency of HCMV in CRC tissue and in normal colonic tissues (p=0.06). However, the mean viral load was 1.78X10⁶±1.7X10⁶ versus 3300 copies CMV DNA per 10⁵ cells in CRC and normal tissues respectively. In colorectal carcinoma, debate exists about the presence of HCMV in tumor tissue. Several studies showed a correlation between HCMV and CRC carcinogenesis (25-31,38,39). While some previous studies showed negative detection of HCMV in the colorectal tumor tissue (22-24). In a meta-analysis study the general prevalence rate of detecting CMV DNA in CRC tissue was 28.7% and explained the lower detection level in FFPE than in fresh frozen tissues due to formalin effect on fragmentation of DNA(40). Also this meta-analysis supported the results of our study in which the frequency of the virus did not differ with the differentiation of the tumor, or in the stage of it (4 in stage 2 and 4 in stage 3), with no significant difference in age or gender which is also in agreement with Dimberg et al in 2013 (26), this study also had 20% and 44% rectal site of tumors versus 10% and 35% colonic site in Swedish and Vietnamese patients respectively, which could support the results of our study in which 7 out of the 8 positive CRC tissues were in the rectum. This finding also can be in agreement with another study done by a member of our research team in 2008 by using immunohistochemistry to detect HCMV antigen in CRC tissues in which 4 out of the 5 positive tissues were rectal site and also there was no significant association with the degree of differentiation (39).

In vitro studies verified HCMV capacity for transforming cells and increasing the rate of tumourigenicity(41). However, HCMV often presents no specific DNA sequence in the transformed cells, which could be explained by “hit and run” mechanism (42,43). Onco-modulatory effect of HCMV permitted to increase the malignancy potential in infected tumor cells by stimulating signal pathways, and transcription factors (20,21). HCMV gene products may also affect the apoptotic potential through activation of PI3K/AKT and Wnt/β-catenin signaling pathways, activation of the signal pathway that are associated with survival and growth of cells, HCMV may increase or enhance cancerous cells’ oncogenic properties (44). If the role of HCMV in CRC carcinogenesis is confirmed, the use of antivirals in cancer progression prevention can be indicated to prevent the proliferation of the virus through antiviral medication (30).

Due to the well-known oncogenic role of EBV in several malignancies, this virus is investigated in CRC tissues. Studies revealed contradictory results about the presence of EBV in CRC tissues (45). The current study revealed no significant difference in the frequency of EBV in CRC (40%), and in the control (20%), which is in line with most of the previous positive studies that gave an EBV positivity rate in ~20-40% of the cases (28,30,46-49). In addition the results of this study are in line with a study in Iran which showed no significant association between the frequency of EBV and patients’ age, sex, cancer site, differentiation or stage (50).

On the other hand, this study found HCMV and EBV DNA in three CRC tissues which is supported by a study of Salyakina et al. (28), which reported a common co-infection of EBV with other viruses in 20% of the CRC samples (CMV, and HHV-6), and EBV and CMV were statistically significantly associated with colorectal cancers when compared to the matched-healthy tissues.

In conclusion, HCMV could play a role in the development of CRC with or without associated EBV infection although their oncogenic role is yet to be established.

Competing Interests:
The authors have declared that no competing interest exists.

Acknowledgement

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