Abstract:
Some epidemiological studies have suggested a possible link between human cytomegalovirus infection and various malignancies particularly colorectal adenocarcinoma.

The current study investigates whether HCMV participates in human colorectal tumorigenesis by the detection of HCMV proteins within epithelial cells of colorectal carcinoma using Immunohistochemistry (IHC). We obtained formalin- fixed, paraffin- embedded specimens of adenocarcinoma, villous adenoma, and normal tissues from the margins of the excision as a control. A total number of 60 specimens were classified into three groups, malignant group, 25 male &10 female, benign group, 10 male & 5 female and control group, 10 specimens. These specimens were tested by IHC to detect the presence of HCMV proteins, early protein (Biogenex, USA) and a mixture of monoclonal antibodies of early and immediate early proteins (Dakocytomation, Denmark). The results of IHC assay showed specific nuclear and cytoplasmic reaction of HCMV proteins within the epithelial cells of colorectal adenocarcinoma (88.5%), and villous adenoma (53.3%), in addition to that no nuclear or cytoplasmic reaction were showed in any case of group C (control). Our data justify further studies to establish whether HCMV interfere with the pathogenesis of colorectal adenocarcinoma.
Introduction:

Despite present understanding of genetic alterations associated with progression of colorectal carcinoma, results of epidemiological studies suggest that environmental factors and host immunological characteristics could contribute to initiation and progression of this cancer. Frequency of colorectal carcinoma is ten times higher in developed countries than in some developing countries, and 3.6 times higher in immunosuppressed patients than in those who are not immunosuppressed.\(^{1,2}\)

The viruses probably play a critical role in carcinogenesis since they are present early during the process of cancer development and are constantly detectable in the tumor cells, such as Epstein – Barr virus\(^{3}\), human herpesvirus – 8 (HHV-8)\(^{4}\), and human papillomavirus (HPV)\(^{5}\); other viruses have been incriminated in human carcinogenesis but there is still a hard debate regarding their direct implication in cancer for example human cytomegalovirus\(^{6}\). In human colonic adenocarcinoma cells, HCMV infection can only arise when these cells are in a specific state of differentiation, virus dose not spread from cell to cell, and productive infection is rare.\(^{7}\) Recently, it was hypothesized that HCMV might be associated with colorectal carcinoma progression; numerous studies had linked HCMV infection with colorectal carcinoma.

Retrospective evaluation of formalin-fixed, paraffin-embedded tissue sections of colorectal adenocarcinoma, villous adenoma, and healthy tissues from margin of excision, for the presence of HCMV proteins in the epithelial cells by using Immunohistochemistry technique.

Material and Methods:

The primary antibody reacts with antigen in the tissue, and then a biotin labeled secondary antibody (link antibody) binds to the primary antibody. When the conjugate is added, the biotinylated secondary antibody will form a complex with the peroxidase – conjugated streptavidin, and by adding the substrate, which contains 3, 3’ – diaminobenzidine (DAB) in a chromogen solution, a brown colored precipitate will form at the antigen site.

Methods:

The procedure applied according to\(^{8,9,10}\):

- Deparaffinization the tissue sections: paraffin-embedded sections were placed inside hot air oven at 70°C, 20 minutes, then immediately dipping in xylene and ethanol – containing jars.
- Preparing retrieval solution 10 x by using coblin jars then put these jars in water bath at 90°C for 20 minutes, left coblin jars to cool for 20 minutes.
- Wash the slides with PBS. Drying and blotting, then put all slides in humidity chamber.
- Aqueous 3% H2O2 was applied onto the tissue to cover the whole Specimen to block endogenous peroxidase for 15 minutes.
- Rinse the slides in PBS, then drying and blotting.
- 50 – 100 µl of diluted primary antibody was applied on the sections for about 30 – 60 minutes.
Apply biotinylated link antibody (secondary antibody) to all sections for 10 – 15 minutes.
Rinse the slides in PBS, drying and blotting.
Apply streptavidin peroxidase (red) to cover the sections for 10 – 15 minutes.
Rinse the slides in PBS, drying and blotting. Apply the prepared DAB-substrate chromogen solution for 10-15 minutes.
Rinse the slides in distilled water.
Dipping the slides in Mayer's hematoxylin as a counter stain for 2 minutes.
Rinse the slides in tap water.
Dipping the slides in the jars containing mixture of tap water (200 ml) and 2 -3 drop of strong ammonia.
Rinse the slides in tap water.
Dry the slides gently, then applied one drop of aqueous mounting media then put cover slip on the slides.
Leave the slides to dry, and then tested.

Preparation of tissue sections and reagents:
1- Paraffin-embedded sections were cut 4 µm in thickness, placed on positive charge slides and left overnight at room temperature to dry.
2- Primary antibody as following:
   A. Anti-pp65 was diluted at 1:50 according to instructions of Manufacture Company.
   B. Mixture antibodies contain two mouse monoclonal antibodies (pp76, pp43), ready to use.
3- Absolute ethanol was diluted in distilled water to prepare 95%, 85% and 75% concentrations of ethanol.
4- Both positive and negative controls were included for each run of IHC; the negative control was obtained by replacing the primary antibody with PBS buffer.
5- DAB chromogen prepare by adding one drop of the DAB chromogen into one ml of substrate buffer.

Evaluation of the immunostaining:
Dakocytomation LSAB2 System – HPR is a highly sensitive system. It is based on a modified labeled avidin – biotin technique, and so, its increased sensitivity was attributed to the small size of the enzyme- labeled streptavidin complex.
The specificity of the immunostaining reaction was demonstrated by the absence of immunostaining in the negative control slides, in both types of monoclonal antibodies the negative control was applied by adding PBS solution to replace primary antibodies, so, the positivity were reported in both positive and negative controls according to the nuclear and/or cytoplasmic reactions.\(^{(11)}\)

Results:
Demography of the patients:
This study involved 35 cases classified as group A (malignant group), their mean ages were \((52.11\pm2.03)\) , 15 cases classified as group B (benign group ), their mean ages \((60.58\pm2.85)\).
There is a significant difference between the mean ages of groups ($p<0.05$), (Table 1).

Table 1: Information about group A and B

<table>
<thead>
<tr>
<th>Variables</th>
<th>Number of cases</th>
<th>Mean Age</th>
<th>Median</th>
<th>Min.Value</th>
<th>Max.Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A*</td>
<td>35</td>
<td>52.11±2.03</td>
<td>52</td>
<td>22</td>
<td>79</td>
</tr>
<tr>
<td>Group B**</td>
<td>15</td>
<td>60.58±2.85</td>
<td>60</td>
<td>39</td>
<td>79</td>
</tr>
</tbody>
</table>

*Group A: Malignant group, **Group B: Benign group ($p<0.05$).

Histopathological data:

From the total of 50 cases (without the controls) of colorectal –tissue samples, there are 35 cases of colorectal adenocarcinoma (70%).

According to the grade of the cancer, these cases classified into 3 grades, GI, GII and GIII.

According to the stage of the cancer, the cases classified into 3 stages (according to the modified astler collar system that applied in the private lab.), B1, B2 & C1 (Table 2).

Table 2: Histopathological data according to the grade and stage of colorectal adenocarcinoma.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients No.=50(malignant group)</th>
<th>Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade</td>
<td>70%</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>Mean</td>
</tr>
<tr>
<td>GI</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>GII</td>
<td>96</td>
<td>48</td>
</tr>
<tr>
<td>GIII</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>B2</td>
<td>34</td>
<td>17</td>
</tr>
<tr>
<td>C1</td>
<td>46</td>
<td>23</td>
</tr>
</tbody>
</table>

Expression of the HCMV proteins:

Clearly positive stains were appeared by IHC technique (Table 3) Group A (malignant group) 31 from the total number 35 (88.5%). Group B (benign group) 8 from total number 15 (53.3%), where as all cases involved with group C (control group) appeared negative. HCMV positive cases showed immunostaining of epithelial cells of colorectal adenocarcinoma and villous adenoma (Figure 1).

The chi-square value with one degree of freedom was found to be 0.028 indicating no significant difference between the positive results of both groups.

Table 3: The results of Immunohistochemistry assay

<table>
<thead>
<tr>
<th>Group A</th>
<th>HCMV positive</th>
<th>HCMV negative</th>
<th>Mean age</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>35</td>
<td>88.5</td>
<td>31</td>
<td>11.4</td>
<td>4</td>
</tr>
<tr>
<td>Group B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>3.3</td>
<td>8</td>
<td>46.6</td>
<td>7</td>
</tr>
</tbody>
</table>
The chi-square with one degree of freedom less than (3.84) are no significant at (p>0.05)

Figure (1): A & B Immunohistochemistry staining of HCMV early protein (brown color) in colorectal adenocarcinoma, grade II, cytoplasmic and nuclear reaction (x400). C & D Immunohistochemistry staining of HCMV mixture of early and immediate early proteins (brown color) in colorectal adenocarcinoma, grade II, cytoplasmic reaction (x800).

Discussion:
Some of studies showed that specific localization of HCMV proteins, early gene messenger RNA and DNA in small series of human colorectal polyps and adenocarcinoma at very high frequency.(11)

Albrech & Rapp first observed that UV-inactivated transformed hamster embryo fibroblast, the specific antigens were demonstrated in the transformed and tumor derived lines, where as HCMV DNA sequences were not detected (12).

A point should be noted here that, The progression through the cell cycle is regulated by cyclins and their associated cyclin-dependant kinases (cdks) which are only active when complexes with their particular cyclin partner,(13,14,15), and progression through the cell cycle occurs as a result of phosphorylation by cdks of specific substrate molecules such as the retinoblastoma –susceptibility protein Rb,(16), other regulatory steps are also involved, these include activation of the cyclin – cdk complex by phosphorylation and
dephosphorylation of specific sites on cdks or inhibition by interaction with so called cdks inhibitors such as p21, p61 and p27.(13,14,15) Generally,high levels of cdk activity are indicative of cell cycle progression, while terminally differentiated or quiescent cells have low levels of cdk activity and elevated levels of mitotic inhibitions.(16) The p53 tumor suppressor protein is a sequence-specific DNA binding transcription factor that transactivates the promoters of many p53-responsive target genes including the cyclin-dependant kinase inhibitors,p21 which mediate cellular growth inhibition, p53 induction can also trigger signals leading to cell death or apoptosis.(17,18) Zhu showed that HCMV infection of human cells block the induction of apoptosis.(19)

Chen and colleagues investigat the mechanism responsible for the decrease of p21 levels after HCMV infection by measuring p21 RNA and protein levels in permissive human lung (LU), by using western blot analysis, they showed, p21 levels declined sharply over the next 24 hours and remained at very low level through 96 hours post infection.(20), on the other hand, Cinalt attempted to investigate whether HCMV infection is associated with reduced TSP-1 production, he found in conjunction with accumulation p53 that TSP-1 mRNA and protein expression were significantly reduced in HCMV – infection cultured human fibroblast.(21)

RB is known to be hypophosphorylated in G0 and early G1 phase of the cell cycle and progression through G1 into S phase is associated with increased RB phosphorylation,(22,23,24), hypophosphorylated RB protein induces cell cycle arrest,(25,26), that's to say, active RB (unphosphorylation form) proteins repress the expression of E2F-target genes by binding to E2F proteins at their C-terminal transactivation domain and recruiting transcriptional repressors to promoters of target genes,(27) and Inactivate RB (phosphorylation form) proteins by cyclin /cyclin –dependent kinase complexes activated during mid-late G1, the RB proteins no longer bind E2F proteins,(28) The E2F proteins play an essential role in regulating the expression of genes required for DNA replication.(29)

On the other hand, the infection of T2 cells (human embryonic carcinoma cell line) results in an increase in the hyperphosphorylated form of RB as early as 24 hours post infection.(16)

Moreover, Bresnahan, have reported that HCMV infection induced cyclin E expression and altered the subcellular localization of a cyclin E-associated kinase, cdk2, in G0 cells resulting in cell cycle progression into the G1 and S phase.(30,31), cdk2-associated with cyclin E is believed to be important for progression through the G1/S phase,(14), cdk2-associated cyclin E kinase activity was maintained in high level through the initial 48 hours of HCMV infection.(16)

Several studies support the idea that the HCMV effects on cyclin protein levels occurred regardless of the cell cycle phase at the beginning of the infection,(32)

Recent analysis by Jault,1995 reported that HCMV infection increase the levels of several cell-cycle regulatory proteins such as cyclins,p53, and phosphorylated RB, particularly he found over expression of both cyclin E and the hyperphosphorylated forms of RB induced by HCMV infection might be the key events initiating the cascade of subsequent modifications of cyclin expression and regulation of the cell cycle,(33), and the accumulation of tumor suppressor proteins including p53 and RB in HCMV –infected cells was associated with loss of their function.(33,34,35,36)
In addition, HCMV infection may also play a role in proliferative diseases such as atherosclerosis\(^{(37)}\) and restenosis\(^{(35)}\). Together, these observations suggest that HCMV was shown to modulate the expression of various proteins involved in cell cycle regulation and apoptosis\(^{(12)}\).

References:

7- Jarvis MA; Wang CE; Meyers HL; et al. (1999). Human cytomegalovirus infection of caco-2 cells occurs at the basolateral membrane and is differentiation state dependent. J. Virol.; 73:4552-60.

