Molecular Targeting of EBERs, EBV- LMP-1 and Cyclin-Dependent Kinases Products in Colorectal Carcinoma Tissues of Iraqi Patients

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Abstract : Colorectal cancers (CRC) have a major toll to healthcare systems worldwide, often fatal cancers with a rapidly increasing incidence. Epstein-Barr virus (EBV) is ubiquitous virus and its role in tumor development and maintenance remains unclear. However, latent membrane protein 1 (LMP-1) of EBV is essential for cellular transformation. Cyclin-dependent kinases (CDK) comprised a family of heterodimeric kinases, playing a central role in the regulation of cell cycle progression, transcription, differentiation and metabolism. To examine the impact of cellular dysregulation mediated by the concordant protein expressions of CDK and EBV- latent genes in implicated in colorectal carcinogenesis. Seventy-five formalin-fixed, paraffin embedded colorectal tissues were enrolled, among them, 60 biopsies obtained from patients with colorectal carcinomas (30 biopsies from the cancer mass and another 30 biopsies from themarginal tissues of these colorectal cancers) and 15 tissues as control group, which were proved by colonoscopic and histopathological examinations as an apparently normal colorectal tissues. Detection of EBERs was done by an ultra sensitive version of in situ hybridization method where asimmunohistochemistry detection system was used to demonstrate the expression of LMP-1& CDK genes.Detection of EBERs-ISH reactions in mass tissues with CRC was documented in 56.7% (17 out of 30 cases), in the colorectal cancer-marginal tissues was 26.7% (8 out of 30 cases), and in the control colorectal tissues constituted 20% (3 out 15). Expression of LMP1 was detected by IHC in 43.4% (13 out of 30 ) of the mass - colorectal cancer group, in 23% (7 out of 30) marginal tissues with colorectal cancer, and in 13.3% of the control tissues. Expression of total CDK-protein was detected by IHC in 17 cases (56.7%) of the CRC- mass group, 11 cases (36.7%) of CRC-marginal group, and 3 cases (20%) in control colorectal tissues group. The differences between the percentages of EBERs-EBV -ISH, LMP-1 and CDK in CRC and control tissues groups are statistically highly significant (P value = < 0.0001). Our results indicate that the significance prevalence of CDK as well as EBV-latent genes - expression in colorectal carcinoma could point to an important contributing role of these molecular and viral factors in the development and carcinogenesis of a subset of colorectal cancers.

Key word : EBERs, LMP-1, EBV,CDK, ISH, IHC.

Introduction
Colorectal cancer (CRC) is considered the third main cause of mortality in the world and it is the most common gastrointestinal cancer and the leading of cancer deaths in the United States of America and western countries (1). Several factors, such as smoking, alcohol use, low rate of fruit and vegetable consumption, obesity, age, family history, red meat consumption, and a lack of physical activity are associated with an increased risk of CRC (2).

The incidence of colorectal cancer varies around the world, where in America, Western Europe, Australia and Japan has the largest rate and in African and Asian countries has the lowest rate (3).

The highly complex pathogenesis of this cancer is involving a sequential genetic and epigenetic mechanisms (4), possibly with a considerable contribution of environmental agents, including bacteria and viruses (5). However, a conflicting results were faced in the search for pathogenic agents, could be related to technical reasons or other unknown factors (6).

Viral etiology in relation with human malignancies is an intriguing issue. All known human tumor viruses contain DNA as their genetic material, with the exception of HCV (7).

The global estimated cancer burden which is associated with oncogenic viruses was 16-18% (8).

Epstein-Barr virus (EBV) is an enveloped, ubiquitous gamma herpes virus with a double-stranded DNA genome encoding more than 85 genes. EBV-mediated growth transformation is characterized by the expression of a subset of viral gene products, including latent membrane protein-1 (LMP-1) and LMP-2 A/B, as well as the nuclear proteins EBNA-1, -2, -3A, -3B, and -3C and LP (9).

These proteins coordinately regulate host signaling pathways to drive resting B cells to proliferate and ensure cell survival by inducing strong anti-apoptotic signals. In addition to these viral proteins, EBV is also expressing several EBV-encoded viral regulatory RNAs, including the EBER RNAs, which are thought to act as inhibitors of host innate immune responses, as well as several virus-encoded microRNAs (miRNAs) (10).

The small untranslated RNAs (EBER-1 and-2) are accumulated at high levels during all forms of latency. EBER-1 interacts with the interferon-inducible protein kinase R to inhibit its activation by double-stranded RNAs, protecting infected cells from INF-induced apoptosis (11).

Virions are periodically shed in saliva of most healthy adults, suggesting one route of viral entry to the stomach is by swallowing, while another route is by hematogenous spread of the occasional infected B lymphocytes that are present in nearly all adults (12).

It is known that EBV infects more than 90% of the world’s adult population. Upon infection, the individual remains a life-long carrier of the virus (13).

Epstein-Barr virus is strongly involved in the pathogenesis of non-Hodgkin’s lymphomas and is associated also with some cases of Hodgkin’s diseases, Burkitt’s lymphoma, gastric and esophageal cancer and rarely with some benign gastrointestinal diseases. A potential role of EBV in colorectal carcinogenesis has also been investigated. So far, studies have provided contradictory results. Some authors were able to detect EBV DNA in colorectal adenocarcinomas by different laboratorial techniques, such as in situ hybridization and PCR (14).

In contrast, others failed to demonstrate the presence of EBV in tissue samples of colorectal cancers, even using a similar methods of detection (15). Although many similar features in histology and pathogenesis between the gastric and colorectal cancers existed, yet few researches have been published in the issue of EBV in relation with colorectal cancers. However, a good bulk of evidences in the support of etiologic role for EBV in carcinogenesis of EBV-positive gastric cancers with a detection rate of EBV ranged from 4 % to 18% (16-19).

The regulation of eukaryotic cell cycle is including a sequential activation and inactivation of several cyclin-dependent kinases (Cdks) to drive cell cycle progression through the phosphorylation and dephosphorylation of such regulatory proteins. Cyclin-dependent kinases [CDKs] were first identified independently in starfish, Xenopus and yeast and cloned in the 1970s–1980s as gene products involved in
regulation of the cell division cycle (20). These serine/threonine proline-directed kinases, which are inactive in their monomeric form, associate with a family of regulatory subunits, cyclins, named after their periodic profiles of expression and degradation, to form functional heterodimeric complexes (21).

In normal cells, these Cdk s are predominantly exist in a quaternary complexes consisting of a Cdk, a cyclin, a proliferating cell nuclear antigen (PCNA) and a 21 kDa protein (p21) . Regarding Cdk activation, it requires cyclin binding as well as the phosphorylation of a conserved threonine residue by Cdk-activating kinase (CAK)(22). The activated Cdk–cyclin complexes can be changed to an inactive state by phosphorylation of a conserved threonine– tyrosine pair or binding to Cdk inhibitory subunits (CKIs). Progression from G1 to S phase in mammalian cells is regulated by the accumulation of cyclins D, E and A, which bind to and activate different Cdk catalytic subunits. The activation of Cdk4–cyclin D and/or Cdk6–cyclin D complex is necessary for transition from early to mid G1 phase. Transition through mid G1 to S phase is regulated by activation of the Cdk2– cyclin E complex. Progression through late G1 to S phase also requires the presence of Cdk2–cyclin A complex (22;23).

To date, twenty different CDKs have been reported in mammalian cells and about the same number of cyclins (24) . Not all CDKs are regulators of cell cycle progression, however, CDK/Cyclins are involved in a wide variety of biological processes, including transcriptional regulation, metabolism, neuronal differentiation and development (25). Since colonic mucosa harbors more chronic inflammatory cells than does gastric mucosa, then the levels of EBV DNA might be different in the two sites depending on the proportion of infected B cells residing there. Therefore, the extent to which EBV levels correlate with levels of inflammatory infiltrate in various tissues could recognize whether EBV is merely an innocent bystander or contributing in a pathogenic fashion to inflammatory lesions. Furthermore, EBV localization in benign epithelial cells could implicate a role for this at an earlier stage of carcinogenesis (26). This study amid to examine the impact of cellular dysregulation mediated by the concordant protein expressions of CDK and EBV- latent genes in implicated in colorectal carcinogenesis.

Materials and Methods

The study was designed as a retrospective one. It has recruited 45 selected formalin fixed, paraffin embedded colorectal tissue blocks; among them, (60) tissue biopsies from colorectal carcinoma with different grades as well as (15) individual healthy colonic tissues (proved by colonoscopic and histopathological examination to be free from any significant pathological changes) were considered as a negative control group for this study. The diagnosis of these tissue blocks were based on their accompanied records. A consultant pathologist reexamined all these cases to further confirm the diagnosis following trimming process of these tissue blocks.

In one hand, the detection of EBV-EBERs by ISH kit (Zyto Vision GmbH. Fischkai, Bremerhaven. Germany) was performed on 4µm paraffin embedded tissue sections using digoxigenin-labeled oligonucleotides probe which targets Epstein-Bar-Virus (EBV) EBER RNA. One section was mounted on ordinary glass slide and stained with hematoxyline and eosin, while another slide was mounted on charged slide to be used for ISH for detection of EBV.

For the in situ hybridization procedure, the slides were placed in 60c hot-air oven overnight then the tissue sections were de-paraffinized and then treated by graded alcohols according to the standard methods and the details of processes for performing ISH reaction with this probe were applied according the instructions of the manufacturing company (Zyto Vision GmbH. Fischkai, Bremerhaven. Germany). The main steps for ISH procedure were: incubation of slides for 18 hr at 70°C on hot plate, then rehydration process was done at room temperature which include: slides immersion in two changes of absolute ethanol for one minute each, then immersion in ethanol (95%) for one minute each, after that immersed in ethanol (70%) for one minute each, finally immersion in a distilled water for 5 minutes to remove residual alcohol. After that, slides were allowed to dry completely by incubating them at 37°C for 5 minutes.

The dewaxing protocols routinely used in immunohistochemistry procedures, e.g. 2-5 min xylene, 2-5 min 100% ethanol, 2-5 min 96% ethanol, 1-5 min 70% ethanol, can be used. Air drying of sections. Then application (drop-wise) pepsin solution to the tissue/cell section and incubate for 20-30 min at 37°C in a humidity chamber. After that we immersed slides in distilled water and drain off the water, air dried sections. Then addition of the probe to the center of a cover slip and placing cover slip upside- down on target area.
Denaturation of the slides at 75°C for 5 min on hot plate, then transferred the slides to a humidity chamber and hybridize for 60 min at 55°C for RNA-targeting probes and the post-hybridization and detection process that included removing the cover slip by submerging in 1x wash buffer TBS, then washed for 5 min in 1x wash buffer TBS at 55°C. Then 20 µl of cDNA probe was added to each section and slides were covered by cover slips with avoiding trapping any air bubbles. After that probe and target DNA were denaturized by placing the cover slipped-slides in pre-warmed oven at 95°C for 8-10 minutes, slides were transferred to a pre-warmed humid hybridization chamber and incubated at 37°C for overnight. Then the slides were allowed not dry out at any time during the hybridization and staining. All reagents used during hybridization and detection were warmed to room temperature. At the next day, slides were soaked in pre-warmed protein block at 37°C until the cover slips fell off and then the slides were allowed to remain in the buffer for 3 minutes, at 37°C after cover slips were removed. Then application of AP-Streptavidin drop wisely to the slides and incubate for 30 min at 37°C in a humidity chamber. Then washed in wash buffer TBS and then twice times for 1 min in distilled water and application. Then one to two drops of Slides were rinsed in detergent wash buffer for 5 minutes and then drained. After that one to two drops of 5-bromo3-chloro3-indoly/phosphate/nitro blue tetrazolium substrate-chromogen solution (BCIP/NBT) were placed on tissue sections. Slides were incubated at 37°C for 30 minutes or until color development was developed completed. Color development was monitored by viewing the slides under the microscope. A dark blue colored precipitate forms at the complementary site of the probe in positive cells. Then the slides were rinsed in distilled water for 5 minutes, then counter staining process by immersion of the slides in Nuclear Fast Red stain for 30 minutes, then washing process was allowed by immersion the slides for 1 minute in distilled water. After that Sections were dehydrated by ethyl alcohol, (95%, once for one minute then, 100% twice times for 2 minutes each); cleared by Xylene, then mounted with permanent mounting medium (DPX).

Immunohistochemistry / Detection system (Abcam. England) was used to demonstrate the LMP-1of EBV &CDK. This technique is based on the detection of the product of gene expression (protein) in malignant and normal cells using a specific monoclonal antibodies, i.e . Primary antibody for specific epitope (usually mouse anti-human monoclonal antibody) , which binds to nuclear targeted protein 37°C for over night.The bound primary antibody is then detected by secondary antibody ( usually rabbit or goat anti mouse ) , which contains specific label ( in this context we used peroxidase labeled polymer conjugated to goat anti- mouse immunoglobulin ) . The substrate is DAB in chromogen solution, positive reaction will result in a browning color precipitate at the antigen site in tested tissues.

Then the slides were dehydrated by immersing them sequentially in the following solution at room temperature for the indicated times, distilled water for 1 minute, 70% ethanol for 1 minute, 95% ethanol for 1 minute and 100% by incubating them at 37% for 5 minutes. After that streptavidin-alkaline phosphatase conjugate reagent was added to tissue sections. Then slides were kept in a humid chamber at 37°C for 20 minutes.Chi –square test was used to detect the significance between variables of our study. All the statistical analyses was done by SPSS program (Version– 17) & P value was considered significant when p <0.05.

Results

I. Distribution of Patients with Colorectal Cancers and Healthy Control Group According to Their Age

The archival specimens collected in this study were related to colorectal cancers patients whom ages were ranged from twenty- one years to eighty five years, where their mean age (53.6± 15.7 years) was higher than the mean age (45.7 ± 11.2 years) of those enrolled in the apparently healthy control. Statistically, no significant difference (p<0.05) was observed between these groups according to the age (Table 1).

Table (1): Distribution of Colorectal Cancers Patients According to Their Age.

<table>
<thead>
<tr>
<th>Maximum</th>
<th>Minimum</th>
<th>S.E</th>
<th>S.D</th>
<th>Mean Age</th>
<th>N</th>
<th>Study Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>85.00</td>
<td>21.00</td>
<td>2.4</td>
<td>15.7</td>
<td>53.6</td>
<td>60</td>
<td>Colorectal Cancer</td>
</tr>
<tr>
<td>75.00</td>
<td>38.00</td>
<td>3.2</td>
<td>11.2</td>
<td>45.7</td>
<td>15</td>
<td>Apparently Healthy Control</td>
</tr>
</tbody>
</table>

(P <0.05) Statistical Analysis
About age distribution; the highest percentage of CRC-occurrence was in the age stratum of 41-50 years (30%), which was followed by the age stratum of 51-60 years (20%), and each of the age stratum of 61-70 years and the stratum of more than 71 year of age (16.7%, each). About gender distribution of the studied patients in relation to their age, the gender of the patients in those who were between 21-30 years was found as (2 male and 2 female), from 31-40 years (4 males and 2 female), 30% from 41-50 years (8 males and 10 females), 20% from 51-60 years (10 males and 2 female), 16.7% from 61-70 years (6 males and 4 female) and 16.7% more than 71 year of age (8 males and 2 female). The highest frequency of male gender was in the age group of 51-60 and the highest female frequency was observed in age group of 41-50 years (Table 2).

Statistically, no significant difference (p< 0.05) was observed between these groups according to the age and gender.

Table (2): Stratification of Colorectal Cancer (CRC) Patients According to Their Age and Gender.

<table>
<thead>
<tr>
<th>Age (Years)</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>21-30</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>6.6</td>
</tr>
<tr>
<td>31-40</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>41-50</td>
<td>8</td>
<td>10</td>
<td>18</td>
<td>30</td>
</tr>
<tr>
<td>51-60</td>
<td>10</td>
<td>2</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>61-70</td>
<td>6</td>
<td>4</td>
<td>10</td>
<td>16.7</td>
</tr>
<tr>
<td>71-</td>
<td>8</td>
<td>2</td>
<td>10</td>
<td>16.7</td>
</tr>
<tr>
<td>Total CRC</td>
<td>38</td>
<td>22</td>
<td>60</td>
<td>100</td>
</tr>
</tbody>
</table>

II. Grading of the studied colorectal cancer

In this study, well differentiated colorectal cancers were seen in 24 cases (40%) including 16 males and 8 females, while 30 cases (50%) (including 18 males and 12 females) have moderately differentiated grade. Poorly differentiated CRC was seen in only 6 cases which comprising (10%) of total CRC group and among them 4 males and 2 female (Table 3). The statistical analysis of grading distribution of colorectal carcinoma shows significant differences (p<0.001) among the grades of Colorectal carcinoma.

Table (3): Distribution of colorectal cancers according to their differentiation grades.

<table>
<thead>
<tr>
<th>Grading of CRC</th>
<th>Gender Male</th>
<th>Gender Female</th>
<th>Total No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well differentiated</td>
<td>16</td>
<td>8</td>
<td>24</td>
<td>40</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>18</td>
<td>12</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>22</td>
<td>60</td>
<td>100</td>
</tr>
</tbody>
</table>

Scoring of the positive EBERs - ISH Signals

Regarding the colorectal cancer -mass group, the total percentage of positive EBERs- ISH detection was 56.7% (17 out of 30 cases), whereas in the colorectal cancer- marginal tissues group was 26.7% (8 out of 30 cases), and EBERs- ISH detection in the control tissues group constituted 20% (3 out 15). Statistically, non significant difference (p>0.05) was found on comparing the percentage of EBERs - ISH reaction-signals between the mass and marginal colorectal tissue groups, while significant difference (p<0.05) was found
between the mass colorectal tissues group and the apparently healthy control group. The highest percentage of EBERs ISH-positive reactions in the mass colorectal cancer group has showed low score (score I) (41.1%: 7 out of 17 cases), while in the marginal colorectal cancer tissues group has revealed moderate score (score II) in (50%) of their examined tissues (Table 4 and Figure 1).

Table (4): Signal scoring of EBERs-ISH reaction-signals.

<table>
<thead>
<tr>
<th>P-value</th>
<th>Apparently Healthy control tissues (n=15)</th>
<th>Colorectal Marginal Tissues (n=30)</th>
<th>Colorectal Mass Tissues (n=30)</th>
<th>EBERs-ISH Reaction-Signals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% No.</td>
<td>% No.</td>
<td>% No.</td>
<td></td>
</tr>
<tr>
<td>0.009</td>
<td>80 12/15 73.3 22/30</td>
<td>43.3 13/30</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 3/15 26.7 8/30</td>
<td>56.7 17/30</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>33.3 1/3 37.5 3/8</td>
<td>41.1 7/17</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0 0/3 50 4/8</td>
<td>35.4 6/17</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td></td>
<td>66.7 2/3 12.5 1/8</td>
<td>23.5 4/17</td>
<td>III</td>
<td></td>
</tr>
</tbody>
</table>

Figure (1): Microscopic appearance of EBERs-EBV-ISH signals of colorectal cancer. Using Biotinylated-Labeled EBERs-EBV-ISH Probe stained with Streptavidin-Alkaline phosphate conjugate (blue) and counter stained with nuclear fast red (red). Blue signal are detected at complementarity sequences sites. A- CRC with negative EBERs-EBV-ISH reactions (40X). B- Positive EBERs-EBV-ISH reaction with moderate score and high signal intensity (40X).

III. Results of LMP1-IHC Signal Scoring

Expression of LMP1 protein was observed as a brownish discoloration at nuclear localization. Expression of LMP1 was detected by IHC in 43.4% (13 out of 30) of the mass-colorectal cancer group, in 23% (7 out of 30) cases with marginal colorectal cancer, and in 13.3% of the tissues in the control group. A high percentage of LMP1 (55%;11 out of 20cases) which have score II were involving cases with mass and marginal colorectal cancer and distributed as (53.9%; 7 out of 13 cases) in the mass colorectal cancer and (57.1%;4 out of 7 cases) in the marginal colorectal cancer, respectively. While, in the control group, 50% (1 out of 2) were found to have in both low score (score I) and moderate score(II). Statistically, significant differences (p <0.05) were found on comparing the results according to positivity of LMP1 expression and their scoring (Table 5 and Figure2).
Table (5): Frequency distribution of immunohistochemical results of LMP1-protein according to the signal scoring of reactions.

<table>
<thead>
<tr>
<th>P-value</th>
<th>Apparently Healthy control tissues (n=15)</th>
<th>Colorectal Marginal Tissues (n=30)</th>
<th>Colorectal Mass Tissues (n=30)</th>
<th>LMP1 Signal scoring</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% No.</td>
<td>% No.</td>
<td>% No.</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>86.7 13/15</td>
<td>76.7 23/30</td>
<td>56.7 17/30</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>13.3 2/15</td>
<td>23.3 7/30</td>
<td>43.3 13/30</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>50 1/2</td>
<td>28.6 2/7</td>
<td>30.7 4/13</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>50 1/2</td>
<td>57.1 4/7</td>
<td>53.9 7/13</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>00 0/5</td>
<td>14.3 1/7</td>
<td>15.4 2/13</td>
<td>III</td>
</tr>
</tbody>
</table>

Figure (2): The Results of Immunohistochemical Staining of Total LMP1-EBV Protein Expression in CRC Using Biotinylated-Labeled Anti-Total LMP1 Protein Antibody, Stained by DAB-Chromogen (Brown) and Counter Stained By Mayer's Hematoxyline (Blue). A. colorectal cancer with negative Total LMP-1 –IHC reactions(40X) B. Positive Total LMP-1 –IHC reaction with low score and high signal intensity (40X).

IV. Results of IHC- Signal Scoring for CDK protein detection

Expression of CDK protein was detected by IHC in 17 cases (56.7%) of the CRC-mass group, 11 cases (36.7%) of marginal group, and 3 cases (20%) in control group. A significant differences (P<0.05) were found when comparing the mass group with its control group. A high percentage of score III (47.1%; 8 cases) were observed among cases in of the mass group. In the marginal group 6 cases (56.5%) revealed score II.(Table 6 and Figure 3).
Table (6): Frequency distribution of immunohistochemistry results of CDK protein according to the signal scoring.

<table>
<thead>
<tr>
<th>P-value1</th>
<th>Apparently Healthy control tissues (n=15)</th>
<th>Colorectal Marginal Tissues (n=30)</th>
<th>Colorectal Mass Tissues (n=30)</th>
<th>CDK protein signal scoring</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% No.</td>
<td>% No.</td>
<td>% No.</td>
<td></td>
</tr>
<tr>
<td>0.009</td>
<td>80 12/15 63.3 19/30 43.3 13/30</td>
<td>20 3/15 36.7 11/30 56.7 17/30</td>
<td>I 66.7 2/3 13.1 3/11 23.5 4/17</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>0.0 0/3 30.4 2/11 47.1 8/17</td>
<td>III 0.0 0/3 30.4 2/11 47.1 8/17</td>
<td>III 0.0 0/3 30.4 2/11 47.1 8/17</td>
<td></td>
</tr>
</tbody>
</table>

Figure (3): The Results of Immunohistochemical Staining of Total CDK Gene Expression in CRC Using Biotinylated -Labeled Anti- Total CDK Protein Antibody, Stained by DAB-Chromogen (Brown) and Counter Stained By Mayer's Hematoxyline (Blue). A. colorectal cancer with negative Total CDK –IHC reactions(40X) B. Positive Total CDK –IHC reaction with low score and high signal intensity (40X).

V. Co-expression of CDK protein with LMP-1 or EBERs

Table (7) shows the association between IHC- expression of CDK protein and the expression of LMP-1 and EBERs of EBV in tissues from colorectal cancer group. The positive results for co-expression of both CDK protein and EBERs- was (%, 10 cases) and (%, 12 cases) for co-expression of LMP1 and CDK protein. However, the association between positive CDK- IHC signal and either LMP-1-IHC or EBERs-ISH positive signals were statistically not significant (P>0.05) in the colorectal cancer group.
Table (7): Relationship between co-expressions of CDK with either LMP-1 or EBERs.

<table>
<thead>
<tr>
<th>EBV Markers</th>
<th>CDK – signal</th>
<th></th>
<th></th>
<th>P- Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>EBERs-EBV</td>
<td>Positive</td>
<td>10</td>
<td>43.5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>13</td>
<td>56.5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>23</td>
<td>100</td>
<td>7</td>
</tr>
<tr>
<td>LMP1-EBV</td>
<td>Positive</td>
<td>12</td>
<td>60</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>8</td>
<td>40</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>20</td>
<td>100</td>
<td>10</td>
</tr>
</tbody>
</table>

Discussion

Colorectal carcinoma (CRC) is a major medical problem worldwide, ranking the third most common cause of death among cancer patients (REF). Only 3-5% of all CRCs are caused by hereditary factors, while the remainder of CRC’s being sporadic. The colorectal carcinogenesis is a multi-step/multi-factorial process, where the association between infections by some bacterial and viral agents with CRC was made since several decades ago (27).

Epstein-Barr virus tends is well known to infect B lymphocytes and epithelial cells as well as been associated with epithelial cell malignancies, such as Burkitt’s lymphoma, nasopharyngeal carcinoma, gastric carcinoma, and post-transplant lymphoma (28).

Epstein-Barr virus (EBV) has been detected throughout the world in about 10% of tissues from gastric carcinoma cases (29). Though colorectal epithelium and colorectal carcinoma are similar to those of gastric epithelium as well as gastric carcinoma, perhaps one reason for the lower prevalence of EBV in CRC as compared to gastric carcinoma might be related to the preferential residence of EBV in the upper gastrointestinal tract tissues compared to the colorectal region and as suggested by (30). However, the role of EBV in the development of CRC still remains as a controversial topic which necessitate further research.

In this respect, the present study was designed to shade a light on the rates of Epstein-Barr virus infections in the microenvironment of CRC- tissues. In the current results, the percentage of EBERs-ISH reactions (56.7%) documented in mass- CRC tissues was more than that percentage in each of marginal tissues of CRC (26.7%) and (20%) in the control colorectal tissues. While, the percentages of expression of LMP1 which was detected by IHC were 43.4%, 23% and 13.3% in the mass- colorectal cancer, marginal tissues of colorectal cancer and the control tissues, respectively.

Comparatively with the obtained results in this study, Sahar et al.,(30) was found EBV-DNA in 60% (9 out of 15) of colorectal tumor samples, and in 40% (14 out of 35) of the non-malignant control group. In addition, Kim et al.,(31) investigated for the presence of EBV in 20 cases of colorectal adenocarcinomas and found 2 cases (10%) were EBER-positive. Moreover, Samahet et al.,(32) and Konet al.,(33) have reported an association of EBV with lymphoepithelioma-like carcinoma of rectum whereas Ruschoffet al .,(34), using polymerase chain reaction, have revealed that EBV may associate differentiated colorectal adenocarcinomas. Furthermore, Grinstein et al., (35) have suggested that EBV might play an oncogenic role in colorectal cancers, and possibly in hyperplasias and certain dysplasias preceding these carcinomas.

Oncogenic proteins identified in this virus are essential for EBV to immortalize B cells and to transform other types of cells, by changing transcription and activation of the cell signaling pathway. These proteins include latent membrane protein 1 and 2 (LMP1 and LMP2), as well as EBV nuclear antigen 2 and 3 (EBNA2 and EBNA3) (36).
The Epstein-Barr virus infection in our series of cases of CRC could express transcripts to activate the proto-oncogene c-Myc, resulting in induction of c-myc on 14q, through the translocation of this proto-oncogene from 8q24 to any locus of the heavy chain of the immunoglobulin to activate the transcription of cellular genes such as CD21 and many other major regulatory viral genes. In addition, EBNAs disrupt cell cycle check points and affect cell cycle progressand then leading to cell damage by affecting various processes such as metabolism, cell cycle regulation, apoptosis, protein synthesis, and angiogenesis, and in line with the aforementioned mechanisms, EBV has potentially privileged to develop various types of cancer (30;36).

Contrary to the many EBV- positive CRC reports, certain papers have suggested a lack of a direct relationship between EBV and the development of such cancers (37). In this respect, Karpinsky and colleagues, and by PCR analysis, found that 19% of the tumor samples were positive for Epstein-Barr virus DNA in 186 sporadic colorectal cancer cases indicating no association of EBV with sporadic colorectal cancers (38). However, many authors have failed to find EBV-related markers in their examined colorectal cancerous tissues, Herein, Boguszakova and colleagues in (39) failed to detect Epstein-Barr viral DNA in the tested biopsies from patients with colonic adenocarcinomas and adenomas. As well, Yuen et al., (40) have investigated Chinese patients for EBV-EBER detection by in situ hybridization (ISH) in 36 cases of colorectal adenocarcinoma, but none of the examined colorectal carcinomas have showed positive signals. Kijima et al., (41) found no positive signals in 102 cases of colorectal cancer using EBER in situ hybridization in Japan. Cho et al., (42) reported no association between EBV with colorectal tumors, too.

Genetic instability is a common feature in many human cancers and seems to play a role in tumor progression leading to cells having growth advantages over normal cells (43). Current understanding has pointed for an approximately 15% of the global cancer burden to be linked to an oncogenic tumor viruses (44). Oncogenic viruses favoring induction of genetic instability and chromosomal aberrations and alternatively their proposed contribution in such carcinogenesis(45).

Tremendous evidences supported an etiologic role for EBV in EBV-positive gastric carcinogenesis. Although there are many similar features in histology and pathogenesis between gastric and CRC, however, there are few articles relating EBV to CRC (46;47).

Our findings showed that the male gender has preponderance in all EBV-positive colorectal tumors. Colorectal cancer incidence and mortality rates are about 35% to 40% higher in men than in women. The reasons for this are not completely understood, but likely reflect complex interactions between gender-related differences in exposure to hormones and risk factors (48).

In a Chinese study, Liu and colleagues have detected EBV DNA by PCR methods, in 26 samples of 130 cases (20%) of colorectal cancers also EBV prevalence among men with cancer than women were diagnosed (49). These results also reflect that age could be an important risk factor affecting colorectal epithelial tissues in favor of tumor changes. In general, the age distribution of the population is considered the most important factor determining the overall incidence of CRC (50). The likelihood of diagnosis of colorectal cancers has increased progressively from the age 40 year with a sharp rising prevalence after the age of 50 years (51). It was noticed that more than 90% of colorectal cancers occurred in people aged 50 year or older (52). The incidence rates have increased more than 50 times in persons aged 60 to 79 years than in patients younger than 40 years (53).

Despite the variability in human oncogenic viruses, yet they shared at least one common feature to efficiently transform the infected cells via targeting important cellular signaling pathways through virus-encoded oncoproteins. These viruses are capable of deregulating tumor suppressor genes; targeting p53 and Rb, various signaling pathways like Notch, JNK, and the cyclin-CDK pathway (54). Among them, the progress of any malignancy that is associated with EBV is via complex cell interactions with specific viral gene expression in the epithelial cells (55).

In the current results, the expression of total CDK-protein was detected by IHC in 17 cases (56.7%) of the CRC- mass group, 11 cases (36.7%) of CRC-marginal group, and 3 cases (20%) in control colorectal tissues group. Proto-oncogene products (such as cyclin D1 and c-myc) in normal cells, act at various levels along the pathways responsible for stimulating cell propagation. Mutated proto-oncogenes can promote tumor expansion while the inactivation of pRb- and p53- tumor suppressor genes, resulting in the dysfunction of proteins and
block cell cycle progression(56). In addition, the dysregulation of cell cycle that associated with cancerous growth occurred typically through the mutation of the proteins that function at various stages of the cell cycle. In human cancers, mutations have been observed in genes encoding cyclins, cyclin dependent kinases (CDKs), CDK-activating enzymes, CDK inhibitors (CKI), CDK substrates and checkpoint proteins(57).

Moreover, EBV-encoded proteins, LMP1 and EBNA3C, have been shown to interfere with p53 functional activity and repress its transcriptional activity(58). In addition, EBNA1 protein interacts directly with cellular de-ubiquitination enzyme, USP7, to regulate the normal p53-Mdm2 pathway, resulting in a reduction of p53 levels, increased cell survival and proliferation (59). Viral oncoproteins as well as E2Fs have been shown to interact through the pocket region of the Rb family of proteins. These pocket proteins are regulated via phosphorylation by CDKs. Hyperphosphorylation of pRb results in loss of binding to both E2F and chromatin remodeling factors and reverses pRb mediated cell cycle arrest (60). As a result, cyclins and pRb family proteins represent key factors for the development of cancer. The high percentage of CDK as well as EBV-latent genes-associated CRC and in our results might indicate for the oncogenic potential of EBV in these cases as well as pointing for its crucial role in development, transformation and/or progression of a subset of colorectal cancers(61-78).

References


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