Molecular Localization of Human Papilloma Virus genotypes (16, 18, and 6/11) in Patients with Colorectal Cancer by DNA- Insitu Hybridization

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Abstract:

Human papilloma viruses (HPV) have been detected in several types of cancers. Over the last few years, a possible correlation between HPV infection and colorectal cancer has been suggested. The aim of this study was to investigate the association between human papilloma virus (HPV) infection and colorectal cancer. To determine the relationship between HPV and colorectal adenocarcinoma, a retrospective study was done. This study was carried out on 50 patients with histopathologically confirmed primary colorectal cancer. Two samples were collected from each patient: one sample from the tumor site and the other one from adjacent normally appearing colorectal tissues, as well as ten (20) colorectal tissues from control individuals with no cancer. In situ hybridization (ISH) was used to detect HPV DNA (HPV 16 and 18 DNA ISH and 6/11 DNA CISH) in colorectal tissues. HPV 16 was detected in 16(32%) of tumor samples, and in 7 (14%) of adjacent normal tissues, and in additionally two cases of apparently healthy group gave positive results for it. HPV 18 was detected in 11 (22%) of tumor samples and in 6 (12%) of adjacent normal tissue. HPV 6/11 was detected in 24 (48%) of tumor samples and in 7 (14%) of adjacent normal tissue. Our results suggest that colorectal HPV infection is common in patients with adenocarcinoma colorectal, albeit at a low DNA copy number, with HPV16 being the most prevalent type. HPV infection may play a role in colorectal carcinogenesis.

Keywords: colorectal cancer, HPV, in situ hybridization.

Introduction:

Papillomaviruses are a group of genetically related organisms, which infect epithelium and infuse proliferation variation in infected cells, which can lead tissues in both benign and malignant tumors (1). Currently, types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 classified as high risk types; types 6,11, 40, 42, 44, 54, 61, 70, 71, 72, 80, and CP6108 are classified as low- risk types; and types 26, 53 and 66 considered as probably oncogenic (2,3). The oncogenic potential of HPV is related to its ability to interfere, upon viral integration into the host cell DNA, with the cell cycle and tumor supportive function of the p53 and pRB proteins (4).

Among non-genital cancers, the association of HPV16/18 infection and oropharyngeal cancer was recently evidenced (5,6,7). However, other non-genital cancers, including lung, breast, and colorectal cancers have not yet been identified (8-13). This is due to a lack of clarity as to how HPV transmits to internal organs even though blood circulation has been suggested as a possible route of infection (14). HR-HPV DNA integration into the host chromosome - regarding predominantly HPV 16/18/31/33/45/53 subtypes - modifies the human epithelial cell DNA by inactivating p53 and Rb gene pathways (15,16).

Colorectal cancer is the third most common cause of cancer-related death in woman and the fourth leading cause of cancer mortality in males. Over 140,000 new cases of CRC is estimated for the U.S. in 2012 with disease-specific mortality of up to 60,000 reported in 2011. Colorectal cancer can be classified as inherited (due to genetic instability), inflamma-
tory (due to presence of chronic inflammation of gastrointestinal tract, e.g., Crohn’s disease) or sporadic, which accounts for more than 80% of all CRC (17).

In the past decade, different scientists have investigated the association between HPV infection and colorectal cancer (18), also colorectal cancer originates in epithelial cells and anal tissues, a site known to be associated with HPV related malignancies, but they have screened completely inconsistent results. Some of the studies reporting 14-84 % of colorectal neoplasia positive for HPV DNA (19). Several studies revealed, HPV has been detected by polymerase chain reaction (PCR) and using in situ hybridization in colorectal cancer (20,21,22), and others detecting slight or no HPV DNA presence in colorectal cancer and adenomatous polyps. Therefore, the association between HPV infection and colorectal cancer is not clear (19).

This study aimed to investigate the presence of HPV 16, 18 and 6/11 DNA by using situ hybridization (ISH) in colorectal cancers in order to determine if a relationship exists between HPV infection and colorectal neoplasm. We next asked whether HPV16, 18, and 6/11 expressed in adjacent normal tissue cells.

Materials and Methods:

The study was designed as a retrospective one. Colorectal tissues were obtained from 50 patients with colorectal cancer as paired tissue specimens by dissection of tumor and tumor-adjacent apparently normal tissues. Specimens belong to the period from June 2010 until November 2013. From each patient two blocks was taken formalin fixed, paraffin embedded colorectal adenocarcinoma (colectomy specimens) and twenty cases from individual colon tissue (proved by colonoscopic and histopathological examination) were proved to be free from any significant pathological changes and were considered as a negative control groups for this study. Mass, margin, and colonoscopy blocks were collected from the archives of histopathology laboratories of Teaching Laboratories of the Medical City/Baghdad and Teaching Al-Hussein hospital/ Kerbala Holy.

The diagnosis of these tissue blocks were based on the obtained pathological records of these cases from hospital files as well as histopathological laboratories records. A confirmatory histopathological re-examination of each obtained tissue blocks was done. Four μm thick sections were made and stuck on positively charged slides. In situ Hybridization / Detection system (Maxim Biotech Inc. USA) used to target DNA sequences using Biotinylated long DNA probe for HPV (16 & 18) and Chromogenic in Situ Hybridization (CISH)/ Detection system (Zytovisions GmbH. Bremerhaven. Germany) used to target DNA sequences using Digoxigenin – labelled long DNA probe for HPV types 6/11. Method was conducted according to the instructions of manufacturing companies leaflet. Positive control reactions were performed by replacing the probe with Biotinylated and Digoxigenin housekeeping gene probe. For the negative control, all reagents were added except the probe. Proper use of this hybridization/detection system gave an intense blue signal at the specific site of the hybridization probe in positive test tissue. Quantification of different molecular markers in situ hybridization signal was evaluated under light microscopy and the counting of positive cells was performed at X1000. In situ hybridization was given intensity and percentage scores, based on intensity of positive signals and number of signals, respectively.

The intensity score included low, moderate, and high intensity of reaction. Positive cells were counted in ten different fields of 100 cells for each sample and the average of positive cells of the ten fields was determined assigning cases to one of the three following percentage score categories: Score(1) = 1- 25%, Score(2) = 26-50%, Score(3)>50% (10). Chi-square test was used to detect the significances between variables of our study. All the statistical analysis was done by SPSS program (version-18). P-value was considered significant when < 0.05.

Result:

The archival specimens collected in this study were related to colorectal cancer patients whom ages were ranged from twenty six years to eighty three years. The mean age of the patients with colorectal cancer (53.54 + 14.8 years) whereas their counterparts apparently healthy control was (46.90 + 12.441 years). There are significant statistical differences (p< 0.05) between different groups according to age (Table 1).

The percentage of sex distribution in the present study, it was found that 31 (62 %) of CRC were males while the rest 19 cases (38 %) were females, Male to female ratio 1.6:1. While the sex distribution in apparently healthy control was found that 6 (60 %) males and 4 (40%) females. The statistical analysis shows significant difference (P<0.05) (table 2).

Regarding mass colorectal cancer group, the total percentage of positive HPV16 – ISH detection was 32% (16 out of 50 cases), whereas in the margin colorectal cancer group was 14% (7 out of 50 cases). In control group HPV 16 DNA constituted 10% (2 out 20). Statistically, non significant difference (p>0.05) was found on comparing the percentages of HPV16 DNA among the mass and margin colorectal tissue groups, while significant differences (p<0.05) was found between margin colorectal tissues and apparently healthy control groups (table 3).

Eleven cases of mass colorectal cancer group (22%) revealed positive signal for HPV18 DNA, margin colorectal group were positive in 6 cases (12%) and control group revealed negative signal for HPV DNA. Significant difference (P<0.05%) was found on comparing the percentages of HPV18 DNA among mass colorectal tissues and apparently healthy control groups but non significant between mass and margin groups (table 4).

The positive results of HPV6/11 DNA in mass and margin colorectal cancer groups and control groups were 24 (48%), 7
(14%) and (0%) respectively. In mass and margin colorectal cancer group high percentages of HPV6/11 DNA were detected in high score 22 (91.7%) and 6 (85.7%) respectively. Significant difference (P<0.05) of HPV6 DNA was found on comparing the positive results according the score among study groups (Table 5).

Histopathological features were studied between positive and negative HPV with mass colorectal carcinomas. The results are shown in (Table 6). It was found the positive results of ISH reactions of HPV16, HPV16, HPV18 & HPV6/11, according to tumor grade of colorectal cancer tissues were found 25%, 31.3%, and 43.8 %, respectively that have well differentiated. While the positive results of ISH reactions of HPV16, HPV18 & HPV6/11, according to tumor grade of colorectal cancer tissues were found 35.5%, 19.4%, and 48.4 %, respectively that have moderate differentiated. Lastly, the positive results of ISH reactions of HPV16, HPV18 & HPV6/11, according to tumor grade of colorectal cancer tissues were found 33.3%, 0%, and 66.7 %, respectively that have poorly differentiated. Statistically, no significant differences (p > 0.05) on comparing the results of HPV genotypes with tumor grade of colorectal cancer.

Anatomic location of the tumor had no correlation with the presence of HPV infection. The highest prevalence of positivity HPV 16, 18, and 6/11 was in left side (7 out of 16 cases), (6 out of 11 cases), and (10 out of 24 cases). Our data showed that HPV16, 18, and 6/11 DNA were present more frequently in stage C, (10 out of 16), (7 out of 11 cases), and (15 out of 24 cases).

Table (1): Distribution of study groups according to their age.

<table>
<thead>
<tr>
<th>Study Groups</th>
<th>N</th>
<th>Mean Age</th>
<th>S.D</th>
<th>S.E</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal Cancer</td>
<td>50</td>
<td>53.54</td>
<td>14.801</td>
<td>2.093</td>
<td>26</td>
<td>85</td>
</tr>
<tr>
<td>Apparently Healthy Control</td>
<td>20</td>
<td>46.90</td>
<td>12.109</td>
<td>2.708</td>
<td>34</td>
<td>75</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table (2): Distribution of study groups according to their gender.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Colorectal Cancer</th>
<th>Apparently Healthy Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Male</td>
<td>31</td>
<td>62</td>
<td>12</td>
</tr>
<tr>
<td>Female</td>
<td>19</td>
<td>38</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>100</td>
<td>20</td>
</tr>
</tbody>
</table>

Table (3): Signal Scoring- Frequency of Positive HPV16 DNA- ISH Reactions.

<table>
<thead>
<tr>
<th>HPV16</th>
<th>Mass Colorectal Tissues (n=50)</th>
<th>Margin Colorectal Tissues (n=50)</th>
<th>Apparently Healthy control (n=20)</th>
<th>P-value1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Negative</td>
<td>34/50</td>
<td>68</td>
<td>43/50</td>
<td>86</td>
</tr>
<tr>
<td>Positive</td>
<td>16/50</td>
<td>32</td>
<td>7/50</td>
<td>14</td>
</tr>
<tr>
<td>Scoring</td>
<td>I</td>
<td>0/16</td>
<td>0.0</td>
<td>1/7</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>7/16</td>
<td>43.8</td>
<td>4/7</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>9/16</td>
<td>56.2</td>
<td>2/7</td>
</tr>
</tbody>
</table>

*P-value2 0.01
Table (4): Signal Scoring- Frequency of Positive HPV18 DNA- ISH Reactions.

<table>
<thead>
<tr>
<th>HPV18</th>
<th>Mass Colorectal Tissues (n=50)</th>
<th>Margin Colorectal Tissues (n=50)</th>
<th>Apparently Healthy control (n=20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Negative</td>
<td>39/50</td>
<td>78</td>
<td>44/50</td>
<td>88</td>
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<tr>
<td>Positive</td>
<td>11/50</td>
<td>22</td>
<td>6/50</td>
<td>12</td>
</tr>
<tr>
<td>Scoring</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1/11</td>
<td>9.0</td>
<td>1/6</td>
<td>16.7</td>
</tr>
<tr>
<td>II</td>
<td>5/11</td>
<td>45.5</td>
<td>4/6</td>
<td>66.6</td>
</tr>
<tr>
<td>III</td>
<td>5/11</td>
<td>45.5</td>
<td>1/6</td>
<td>16.7</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>HPV16/11</th>
<th>Mass Colorectal Tissues (n=50)</th>
<th>Margin Colorectal Tissues (n=50)</th>
<th>Apparently Healthy control (n=20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Negative</td>
<td>26/50</td>
<td>52</td>
<td>43/50</td>
<td>86</td>
</tr>
<tr>
<td>Positive</td>
<td>24/50</td>
<td>48</td>
<td>7/50</td>
<td>14</td>
</tr>
<tr>
<td>Scoring</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0/24</td>
<td>0.0</td>
<td>0/7</td>
<td>0.0</td>
</tr>
<tr>
<td>II</td>
<td>2/24</td>
<td>8.3</td>
<td>1/7</td>
<td>14.3</td>
</tr>
<tr>
<td>III</td>
<td>22/24</td>
<td>91.7</td>
<td>6/7</td>
<td>85.7</td>
</tr>
</tbody>
</table>

Table (6): Colorectal Cancer and Patient’s Histopathological Characteristics According to HPV DNA Presentation.

<table>
<thead>
<tr>
<th>Histopathological Characteristics</th>
<th>HPV16</th>
<th>HPV18</th>
<th>HPV6/11</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer Grading</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Well differentiated</td>
<td>4</td>
<td>12</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>11</td>
<td>20</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Site of tumor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right side</td>
<td>6</td>
<td>11</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>Left side</td>
<td>7</td>
<td>16</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>Rectum</td>
<td>3</td>
<td>7</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Stage of tumor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>10</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>19</td>
<td>7</td>
<td>22</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>
Discussion:

In the last few years, a possible correlation between HPV infection and colorectal cancers and adenomas has been suggested (23,24,25) but results are mismatch. Some studies reporting HPV-DNA in various prevalence in colon cancers and adenomas, (20,21) but another researches investigations failed to establish a link between HPV and colon malignancy (26,8). Therefore, the possible role of HPV infection in colorectal carcinogenesis is still a subject of great controversy.

Demographic data including age and sex in patients with colorectal carcinoma in present study are comparable to reported by Boyle and Leon (2002), Haggard and Boushry (2009), Almeida and Barry (2010), Reza et al (2014), and Eman et al (2014), which indicate that more patients of colorectal cancer are older than 50 years of age and the cancer incidence rate among men is higher than women (27-31).

In the present study, HPV DNA 16 and 18 were detected in (32%) and (22%) respectively of mass colorectal carcinoma and of match controls in margin colorectal tissues and colon tissue of apparently healthy control we found significant evidence between CRC and HPV infection in the role of HPV infection between CRC and control group in the analyzed samples. This is in keeping with the findings of Bodaghi et al. (2005), Damin et al., (2007), Motlagh et al,(2007), and Chen et al, (2012) (20,22,32) but some have investigating non-significant role of incidence HPV infection in colorectal carcinoma (8,19,33).

HPV 6/11 were detected in 24 cases of mass colorectal tissues and 7 cases in the adjacent normal tissue samples. This results agreement with many studies (24) investigated the presence of HPV 6, and HPV 11 were detected in 8, 22, 32 cases respectively in CRC patients and found significant differences between mass colorectal tissues and adjacent normal tissue samples. Salepche et al (2009), used PCR and Southern blot for the detection of HPV in CRC patients. They detected HPV 6/11, 16/18 and 33 in 82% of tumor samples and 32% of adjacent normal tissue samples (p<0.001) (25).

Liu et al. investigated the prevalence of HPV in a Chinese population suffering from colorectal cancer. Their results showed that the frequency of HPV DNA found in tumor tissues was higher than in non tumor colorectal tissues and peripheral blood samples (P < 0.001) (34).

Gillison and Shah (2003), indicated that HPV associated malignancies would occur at anatomic subsites of exposure by direct contact, since there is no viremic phase in the pathogenesis of HPV, so the infection is not widely disseminated in the body (35). On the other hand, Bodaghi et al. (2005) reported that HPV infection in the tumor tissues which obtained from the cecum and ascending is as common as in the tissues obtained from rectosigmoid locations and this indicated the HPV infection might not be a result of the direct spread from anogenital sites (20), also Chen et al. (2012) found that the transmission of the HPV to the colorectal tissues might occur through blood circulation (18). Since higher frequency of HPV infection in cecal and ascending tissues has been determined in compare to frequency of infection in rectosigmoid tissues, this study supports the hypothesis that investigated HPV infection in colon and rectum might not occurs through direct infection. Magalhães et al, 2014, which that found was not any significant difference in relation of HPV infection to degree of cell differentiation among the patients with colorectal cancer (36).

Many authors in analyzing the cancer group regarding the presence or absence of HPV in relation to tumor location were not found any statistical differences in these variables (20,22). In recent study, also no significant relationships between the virus’s presence and tumor location were found in studies carried out by Miąsko et al, 2012; and Deschoolmeester et al, 2010, these result agreement with current study (37,38).

According to Motlagh et al, 2007, HPV DNA presentation was not significantly associated with including tumor stage, this result are likewise to result of current study. One the other hand, Moreas et al, 2014, identified the presence of high frequency of HPV DNA Dukes A-B stage with statistical significant, while in present study more frequency of HPV DNA in stage C with non statistical significant, these incompatible with previous study (32).

Despite some suggestive studies for the presence of an association between HPV and CRC, infection with HR-HPV seems to be not sufficient by itself to cause cancer. Additional cellular alternations are necessary for tumorigenesis and an accumulation of mutations appears to occur over time (2).

In summary, HPV infection seems to be common in colorectal cancer tissues and adjacent non-tumor tissues, suggesting that HPV might play a role in the pathogenesis of colorectal cancer.

References:


الموضوع الجزيئي للفيروس الحليمي البشري ذو الأنماط الجينية (16 و11 و 6 و 18) في مرضى سرطان القولون والمستقيم باستخدام تقنية الدنا التهجين الموضعي

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3 كلية الطب – جامعة بغداد
4 المختبرات التعليمية – مدينة الطب

الخلاصة:

تم الكشف عن فيروسات الورم الحليمي البشري في عدة أنواع من السرطانات. على مدى السنوات القليلة الماضية، وقد اقترح وجود علاقة محتملة بين عدوى فيروس الورم الحليمي البشري وسرطان القولون والمستقيم. هدفت الدراسة الحالية لتحري عن العلاقة بين الإصابة بفيروس الحليمي البشري وسرطان القولون والمستقيم. قصمت هذا الدراسة كبحث ذو أثر رجعي، إذ اشتملت على (50) مريض مع اجراء الفحص النسيجي لتشخيص سرطان القولون والمستقيم. تم جمع عينات من كل مريض: عينة واحدة من موقع الورم والآخر من الانسجة المجاورة، بالإضافة إلى عينة من أنسجة القولون والمستقيم من أفراد غير مصابين ظاهرا كمجموعة سيطرة لهذه الدراسة. استخدمت تقنية التهجين في انسجة سرطان القولون والمستقيم. ظهرت نتائج الدراسة أن فيروس الحليمي البشري نمط 16 و18 و6 لم يظهر في الأنسجة الطبيعية المجاورة إلا في حالة واحدة (0.7%) من عيّنات الورم حيث كان النتائجimbledon (32%) من عيّنات الورم بينما في الأنسجة الطبيعية المجاورة كانت 7 حالة ونسبة (22%) ونسبة (14%)، وحالتلان فقط من مجموعه السرطانات أظهرت نتائج إيجابية. اكتشف الفيروس نمط 16 و18 في 11 حالة ونسبة (22%). من الورم ونسبة (14%) من النسج الطبيعية المجاورة. الفيروس الحليمي البشري نمط 11 و1/6 و1/18 اكتشف في 5 حالة ونسبة (10%) من النسج الطبيعية المجاورة. تتفق نتائج الدراسة أن الإصابة بفيروس الحليمي البشري شائعة في مرضى سرطان القولون والمستقيم، ولو أنه عدد الدنا المضاف، فيروس الحليمي البشري نمط 16 النوع الأكثر شيوعا. الإصابة بفيروس الحليمي البشري قد تلعب دور في تكون سرطان القولون والمستقيم.