Molecular Targeting of *John Cunningham* Virus (*JCV*) Among Colorectal Tumors Patients in Central Iraq

Zahraa Ali Abdullah **Shakir Hammad Mohammed**
College of Sciences , Babylon University
alizahraa128@yahoo.com. shakir_immuno@yahoo.com

Abstract

In order to prove the implication of *John Cunningham* virus in colorectal cancer of Iraqi patients. Sixty eight (68) formalin-fixed, paraffin embedded colorectal tissues were obtained in this study ;42 biopsies from colorectal carcinoma (CRC) and (16) from benign colorectal tumors as well as (10) apparently normal colorectal autopsies control group. The age of these individuals (patients and control groups) were ranged between 7 and 85 years. The patients samples were collected from the archives of histopathology laboratories of AL-Shaheed Gazi Al-Hariery Hospital for Specialized Surgery /Baghdad Teaching Hospital in Baghdad Medical City ;Al-Hilla ;AL-Saddar(Al-Najef); Al-Hussein (Kerblla) as well as many private histopathology laboratories that generously helped as and are kindly thanked in the present dedication. This study found the percent of *JCV*s -ISH in tissues with CRC observed in42.8% (18 out of 42 cases), and in the benign was detected in 6.25% (1 out of 16 cases) while, in the healthy control group was detected in10% (1 out of 10 cases). The highest rates of *JCV* detected in relation with tumor grade depending on the differentiated of cells were 61.1 % (11 out of 18 cases) in grade I(well differentiated carcinoma), followed by 33.3% (6 out of 18cases) in grade II(moderately differentiated carcinoma), and 6.55% (1 out of 42 cases) in grade III (poorly differentiated carcinoma).

Conclusion of This Study

Significant association of *John Cunningham* virus infection with colorectal cancers indicate for an important possible role for this viral agent in the development of this subset of colorectal tumors.

Keyword: *JCV*; Colorectal tumor; *In Situ* Hybridization.

الخلاصة

صممت هذه الدراسة كبحث ذو أثر رجعي (Retrospective study) اذ اشتملت على ثمانية و ستون (68) خزعة نسجية من منطقة القولون (colorectal) المحفوظة بالفورمالين والمطمورة بشمع البارافين . اذ تضمنت اثنان و اربعون (42) عينة اخذت من مرضى مصابين بسرطان القولون وستة عشرة (16) عينة اخذت من مرضى مصابين بورم القولون الحميد. كما ادخلت عشرة (10) قطع نسيجية من عينات القولون غير المصابة ظاهريا كمجموعة سيطرة لهذه الدراسة . كان عمر الاشخاص في مجموعتي المرضى والسيطرة يتراوح بين 7 – 85 سنة.

جمعت هذه النماذج من أرشيفات الأنسجة المرضية لمختبرات مستشفى الحلة التعليمي/بابل , مستشفى الصدر التعليمي/ النجف الاشرف ,مستشفى الحسين التعليمي/كربلاء المقدسة و مستشفى الديوانية التعليمي/الديوانية , بالإضافة الى العديد من مختبرات الأنسجة المرضية الخاصة الموجودة في بابل, النجف, كربلاء, و أرشيف معهد الطب العدلي في بابل . تم جمع هذه العينات للفترة من آب 2010 الى شباط 2014 . وتمت مطابقة العمر والجنس لكل عينة من هذه العينات.

شكلت مجموعةِ العينات المحفوظة لسرطان القولون والتي أظهرت نتائج موجبة لفايروس الجون كنينكهام (*JCV*) نسبة % (18 42.8من اصل 42 عينة) من مجموع هذه العينات, ووجدت بنسبة 6.25 % (1 من اصل 16 عينة)في مجموعة اورام القولون الحميدة .لا توجد حالات موجبه لفايروس الجون كنينكهام (*JCV*) في مجموعة انسجة قولون الاصحاء الضابطة. تم تحديد اعلى نسبة للإصابة بفايروس الجون كنينكهام (*JCV*)–في مجموعة العينات السرطانية اعتمادا على مراحل تمايز الخلايا في مرحلة الخلايا غير المتمايزة حيث بلغت % 61.1(11 من اصل 18 عينه),يليها 33.3%(6 من اصل 18 عينه) في مرحلة متوسطة التمايز ,واخيرا 6.55%(1 من اصل 18 عينه) في عينات السرطان للخلايا عالية التمايز.

الكلمات المفتاحية: فايروس الجون كنينكهام (*JCV*) ، سرطان القولون

Introduction

Colorectal cancer is amongst the most common malignancy found in the Western world , usually ranks high in incidence and mortality among malignancies in these countries (Parkin *et al*.,2005). Globally, colorectal cancers accounted for about 1 million new cases in 2002 (9.4% of the world total). In terms of incidence, colorectal cancers rank fourth frequency in men and third in women. The main pathology type of the colorectal cancer is adenocarcinoma, though some other variants can occur (Lam *et al*.,2006).

John Cunningham Virus (*JCV*) is a human neurotropic polyomavirus, and neurological diseases, such as progressive multifocal leukoencephalopathy have been associated to *JC* virus . JCV is a virus very well adapted to humans, thus its widespread infection and adaptation to humans complicates the determination of its etiologic contribution to cancer development, and it has also been associated to some neurodegenerative diseases (Jiang, *et al*.,2009).

JCV DNA sequences and proteins have been detected in a broad range of human tumors of glial and non-glial origin, including gliomas, ependymomas and medulloblastomas, as well as in several non-neural clinical specimens of upper and lower gastrointestinal tumors, such as colorectal cancer (CRC) (Burnett-Hartman *et al*.,2008), suggesting they can infect a wide range of cell types.

The variability in *JCV* detection suggests that in an infected colon, in some cells there might be integration with partial loss of *JCV* DNA, which may have a pathogenic role in cancer development, probably permitting additional events that will lead to cancer progression by permitting selection of a cell subpopulation. When human CRC samples were grown as xenographs in nude mice that permit expansion of the cancer cell population, all of them resulted positive for *JCV* (Laghi *et al.,*1999*)* ,suggesting that the cell subpopulation containing *JCV* might be selected for its growth and adaptation characteristics.

Materials and Methods

The study was designed as a retrospective one. It has recruited 68 selected formalin fixed, paraffin embedded colorectal tissue blocks among them; (42) tissue biopsies from colorectal carcinoma with different grades and (16) benign colorectal hyperplastic tissue blocks as well as (10) apparently normal colorectal tissue autopsies which were collected from the archives of Forensic Medicine Institute / Baghdad and used as colorectal healthy tissues control groups. 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 Situ Hybridization technique (ISH).

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 JCV .The detection of JCV-DNA by ISH kit (Zyto Vision GmbH. Fischkai, Bremerhaven. Germany) was performed on 4μm paraffin embedded tissue sections using Biotinylated-labeled oligo-nucleotides probe which targets (JCV) DNA.

For the in situ hybridization procedure, the slides were placed in 60 C˚ hot-air oven over night then the tissue sections were de-paraffinized and via then incubation of slides for 15 min (twice time) in xylene then treatment by graded alcohols via

incubation for 5 min in 100% ethanol(twice time) , 5 min 96% ethanol(one time), 5 min 70% ethanol(one time), were used, finally immersion in 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. Then was occurred digestion process by add pepsin solution (ES1) to the slides, then the slides were incubated at 37°C for 20-30 minutes in humidity chamber. Then the slides immersion in distilled water for 5 minutes to remove pepsin solution 10 µl of (*JCV*) DNA probe were added to each section and slides were covered by cover slips be careful to avoid trapping any air bubbles. After that probe and target DNA were denaturated by placing the cover slipped-slides in pre-warmed oven at 75°C for 8-10 minutes, slides were transferred to a pre-warmed humid hybridization chamber and incubated at 37°C for overnight. 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 wash buffer at 37°C until the cover slips fell off and should be careful not to tear the tissue, then the slides were allowed to remain in the wash buffer for 3 minutes, at 37°C after cover slips were removed. After streptavidin-alkaline phosphatase conjugate reagent were added to tissue sections. The slides were kept in a humid chamber at 37°C for 20 minutes. 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 tertrazolium substrate-chromogen solution(BCIP/MBT) were placed on tissue section. 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 form at the complementary site of the probe in positive cells. These 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 seconds, then washing process was followed 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).

Statistical Analysis

Chi –square & phi test was used to detect the significance of variables in our study. All the statistical analysis was done by SPSS program (Version– 20) & P value was considered significant when $p < 0.001$.

Results

The Result of *JCV*-DNA by *In Situ* Hybridization Technique (ISH).

Results of *JCV*-DNA - ISH Signal Scoring:

The John Cunningham Virus score signaling was detected in tissue blocks obtained from patients with malignant colorectal tumor, benign colorectal tumor, and healthy colorectal tissue. The score signal of *JCV*-ISH was detected as blue discoloration at the site of complementary sequences in the nuclear region. Figures (1,2& 3) shows the positive result of *JCV*-ISH detection where 42.8% (18 out of 42 cases) from malignant group showed positive signals included (66.6%: 12 out of 18 cases) in the weak score (score I),followed by (16.7%: 3 out of 18 cases) in both the strong score (score III),and moderate score (score II). The benign group revealed 6.25% positive signals which represented (1 out of 16 cases) in this group

None of control group presented positive signals for *JCV*-ISH test. . The Statistically analysis showed medium significant differences depending on (Chi-square & Phi test) in p>0.001.

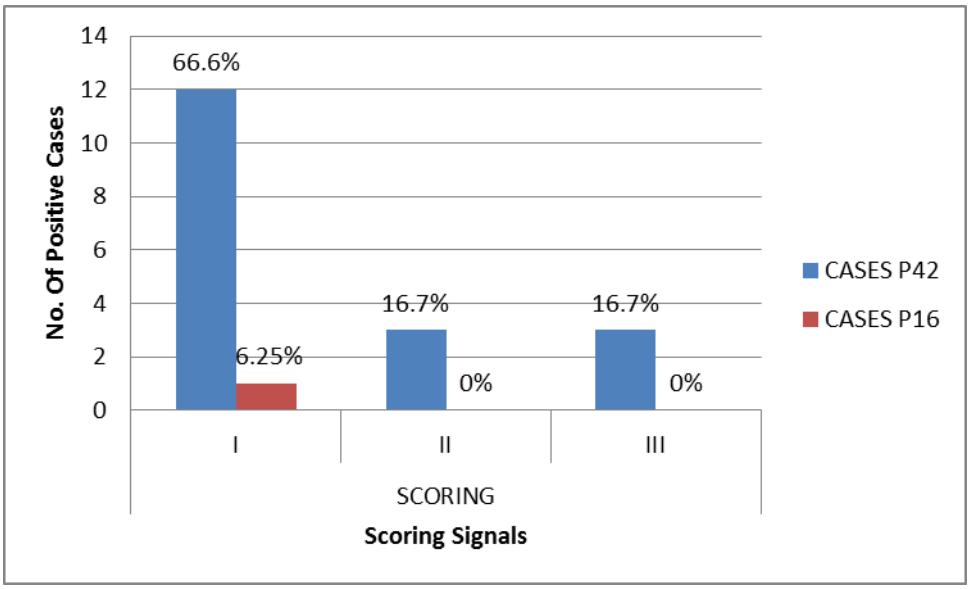


Figure 1: The percentage of *JCV* score signaling in malignant, benign nasopharyngeal tumor, and healthy colorectal tissue. (Chi-calculated = 0.952381, Chi-table = 18.4668 , Phi = 0.218)
P42=malignant cases P16=benign cases P10=control cases
I=weak score II=moderate score III=strong score
Results of *JCV*-DNA- ISH Signal Intensity

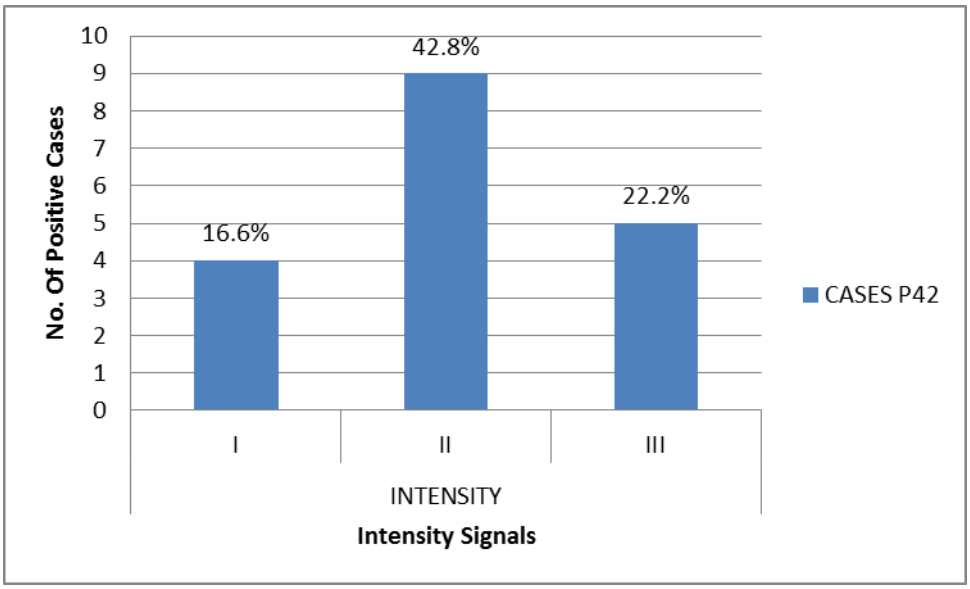
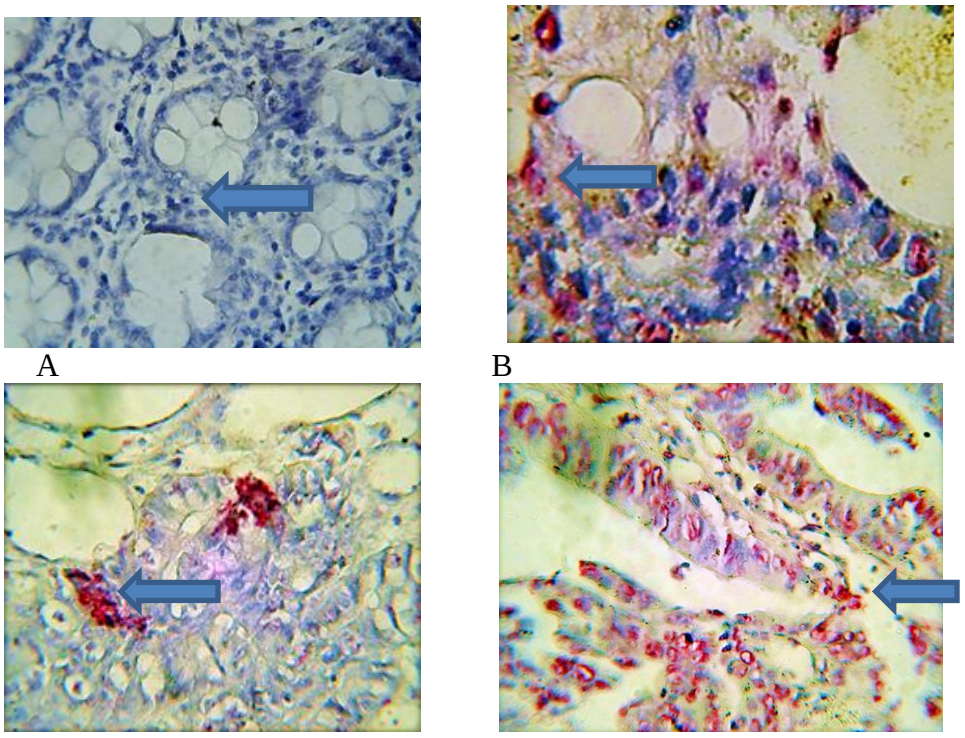


Figure 2: The percentage of *JCV* intensity signaling in malignant, benign colorectal tumor, and healthy colorectal tissue. (Chi-calculated = 1.818182, Chi-table = 18.466, Phi = 0.302)
P42=malignant cases P16=benign cases P10=control cases
I=weak intensity II=moderate intensity III=high intensity

The *John Cunningham* Virus intensity signaling was detected in tissue blocks obtained from patients with malignant colorectal tumor, benign colorectal tumor, and

healthy colorectal tissue. The intensity signal of *JCV*-DNA-ISH was detected as blue discoloration at the site of complementary sequences in the nuclear region. Figures (2, 3) shows the positive result of *JCV*-DNA-ISH detection where 42.8% (18 out of 42 cases) from malignant group showed positive signals included 50% (9 out of 18 cases) in the moderate signal intensity (II),
followed by 22.2% (4 out of 18 cases) in the high signal intensity (III), and 16.6% (3 out of 18 cases) in the low signal intensity (I) . The Statistically analysis showed, medium significant differences depending on (Chi-square & Phi test) in p>0.001.



A B

C D

Figure 3*: In Situ* Hybridization (ISH) for *JCV* Detection of Colorectal Cancer Using Biotinylated-Labeled *JCV* Probe; Stained With NBT/BCIB (Blue) and Counter Stained by Nuclear Fast Red (RED) .
A-Colorectal cancer with negative *JCV*s-ISH Reaction (40x)
B-Positive *JCV*s -ISH Reaction with Strong signal score and High signal intensity (4x)
C- Positive *JCV*s -ISH Reaction with Moderate signal score and Moderate signal intensity (10x)
D- Positive *JCV*s -ISH Reaction with Low signal score and Weak signal intensity (40x).

The Result of *JCV* s in Malignant Colorectal Tumor According to the Grades of Patients:

The *John Cunningham* Virus- DNAs (*JCV*-DNA) was detected in tissue blocks obtained from patients with malignant colorectal tumor . The signal of *JCV*- ISH was detected as blue discoloration at the site of complementary sequences in the nuclear region. Figure (4) shows the positive results of *JCV* DNA-ISH detection ,where 42.8% (18 of total 42) from malignant group showed positive signals included

61.1.5% (11 out of 18 cases) in well differentiated carcinoma grade, followed by 33.3% (6 out of 18 cases) in moderately differentiated carcinoma grade, and 5.6% (1 out of 18 cases) in poorly differentiated carcinoma grade. The statically analysis showed significant difference between grade of colorectal tumor (P value >0.001).

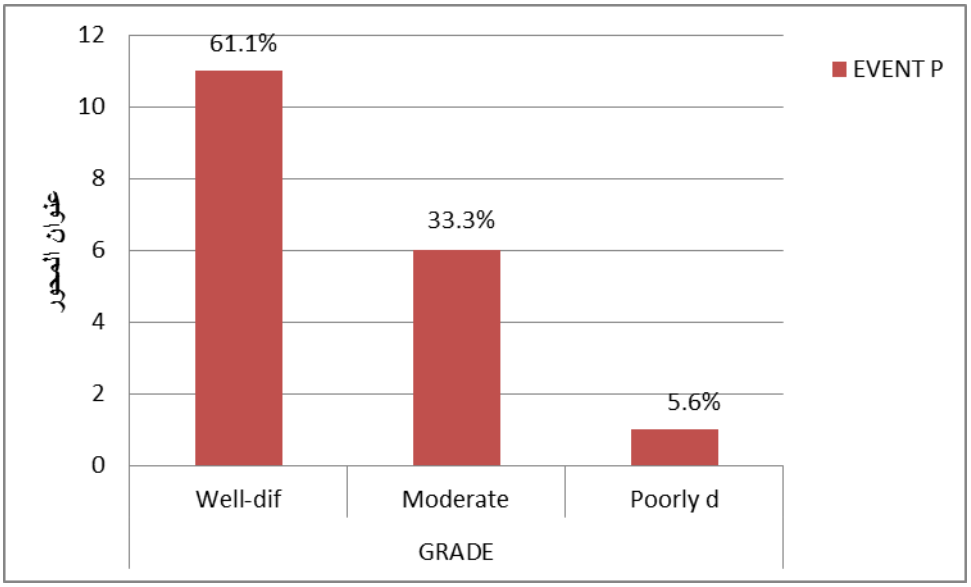


Figure 4 : The positive & negative result of JCVs in patients according to the grades of malignant colorectal tumor (Chi-calculated = 0.966, Chi-table = 13.815, Phi = 0.151697)

Discussion

Several studies have demonstrated that *JC* virus infection takes place during early childhood and remains subclinical. After primary infection, *JC* virus can be found in the kidneys, B lymphocytes, and gut mucosa (Sinagra *et al.* 2014).

The first reports suggest that JC virus can play role in human CRC date from 1999.Shortly after this, more studies have reported an association of *JC* viral infection with CRC (Antonic *et al*. 2013).

However ,up to our knowledge this study of *JCV* in colorectal cancer is the first research work in Mid-Euphrates Governorates of Iraq ,that was designed to analyze the association of *JCV* in colorectal tumors by using the technique of *in situ* hybridization (ISH).

In the present study ,the *JCV*- DNA percentage in malignant colorectal tumors (42.8%) was higher than its percentage (6.25%) in their benign counterparts. This finding reflects a possible role of the *JCV* in the carcinogenesis of colorectal malignant tumors group or may have -precancerous effect in benign group. Also, may be could in turn indicate for a respective role of this virus in the colorectal cancers pathogenesis and/or their multi-step carcinogenesis.

The negative results are probably related to the absence of *JCV*-DNA in these biopsies or could be related its presence in the cells at different regions of that tissue.

Sinagra, *et al*., (2014) reviewed five studies examining colorectal neoplastic tissue detecting *JC* virus DNA in colorectal neoplasias at varying frequencies, finding from 26% to 89% of carcinomas positive for *JC* virus. This results was consistent with current study.

Sinagra, *et al*., (2014) was presented lower rates of detection for *JC* virus, finding 26% of cancerous colorectal tissue and 0% of normal tissue positive for *JC* virus. These results were agreement with our results.

Casini *et al*.,(2005) reported that 16 out of 18 patients (88.9%) have been positive for the presence of JCV DNA, assessed with three techniques, PCR, Northern blot and in situ hybridization, within the primary tumor mass and peritumoral tissue . This results was consistent with our study.

A study from Taiwan, Antonic *et al*., (2013) was conducted on formalin-fixed, paraffin-embedded tissues from 22 colon cancer patients, identified genomic DNA in 86.4% (19/22) of the CRC tissue samples. This study also identified expression of viral early protein, but not structural capsid protein, in the examined colon cancer tissues. *JCV* T-Ag DNA sequences were found in 77% of CRCs studied; and 56% of these cancers (or 43% of the total) expressed T-Ag by IHC. This results was compatible with current study.

Ricciardiello *et al*., (2004) was reported a high prevalence of *JC* virus in gastric and colonic tissue from patients without gastrointestinal neoplasia (>70% of participants).This result agreement with current results.

Expression of the viral oncogenic early protein, T-antigen, and the late auxiliary protein, Agnoprotein, was observed in >50% of the samples, also showing that *JCV* can interact with β-catenin, which in turn dysregulates the Wnt pathway and finally the c-myc promoter (Enam *et al*., 2002)

Our results disagree with Enam *et al*. (2002) reported, neither their data nor others support a role of *JC* virus as a cause of colon cancer (Boland *et al.*, 2004).

The variability in *JCV* detection suggests that in an infected colon, in some cells there might be integration with partial loss of *JCV* DNA, which may have a pathogenic role in cancer development, probably permitting additional events that will lead to cancer progression by permitting selection of a cell subpopulation. When human CRC samples were grown as xenographs in nude mice that permit expansion of the cancer cell popula tion , all of them resulted positive for *JCV* (Coelho, *et al*.,, 2010), suggesting that the cell subpopulation containing *JCV* might be selected for its growth and adaptation characteristics.

Colorectal cancers can show chromosome instability and it was hypothesized that JCV may account for some of this instability.Therefore, if this virus is present in colon tissue, it would be integrated in the genome and not be in a superhelical form. Thus, topoisomerase treatment would be unnecessary. Furthermore, the DNA was isolated from tissue blocks, which yield highly fragmented DNA. Despite the relatively low DNA quality, 10 microsatellite markers in single-copy genes have been successfully amplified from all the tissue samples (Newcomb *et al.*, 2004).

JCV transient effects might lead to selective expansion of tumor cells. Since there is not a direct cause and effect relationship, *JCV* infection may be an alternative to low frequency cancer pre- disposition genes (Coelho *et al.*, 2010).

The dilemma of presence and/or transmission of *JCV* to colorectal tissues is still meticulously studied yet ,the transmission routes of *JCV* that were detected in colorectal cancer are not yet unclear.An appreciation of the mechanisms of viral transmission, sites of latency, re-expression, and trafficking into the brain are fundamental to understanding the pathogenesis of progressive multifocal leukoencephalopathy (PML). The absence of an animal model for PML has precluded answers to these fundamental questions. (Berger et al. 2006).

Some of the new HPyVs may transmit horizontally by direct contact, or transmit by aerosol or fecal–oral routes (Feltkamp *et al*., 2013). Infection site, the original site of

the polyomavirus's identification, and the distant organs in which the virus is detectable may, however, be distinct and be hard to relate to any pathophysiology (Dalianis *et al.,* 2009).

In situ hybridization methods for detection of nucleic acid sequences have especially proved powerful for revealing genetic markers and gene expression in a morphological context (Kenny *et al.*,2002). ISH techniques are also effective methods to detect and localize Viral DNA within the affective tissues using formalin-fixed and paraffin-embedded (FFPE) clinical samples. This technique allows histopathologists a direct comparison between the histological features and the viruses status at sensitivity similar to that of southern blot hybridization and PCR (Huang and Pan *et al.,* 2005).

The high frequency of *JCV* excretion in urine suggests the kidney as a site of viral latency; however, DNA sequences of the regulatory region of kidney or urine isolates are markedly different from those in the brains of patients with PML (Berger *et al., 2006).*

Other studies believed that viruses excreted in urine and feces are transmitted through what is known as fecal contamination, which includes viruses excreted in feces and urine. Our findings of high levels of *JCV* and *BKV* in most sewage samples and the relative stability of these viruses under environmental conditions suggest that the alimentary tract could be an important point of exposure and transmission of these viruses among humans (Bofill *et al*., 2001).

The positive results of ISH reactions of *JCV*, according to tumor grade of colorectal cancer tissues was found 61.1 % in well differentiated followed by 33.3% in moderately differentiated carcinoma grade, and 5.6 % in poorly differentiated carcinoma grade .

On analysis of the results in the Figure 4-9, it was noticed a decreasing percentage of *JCV*-infection with the progressing of the cancer grade to the worse one. On matching each score (from I to III) with each counterpart cancer grade (from well differentiated grade towards the worse one) it was noticed an decreasing trend of correlation of the *JCV* signal (in referring to worsening of this disease).

JCV was more frequently detected in tumors of well differentiated morphology (in 44 of 66 cases; 66.7%) in contrast to tumor of poor differentiated type (in 3 of 11 cases; 27.3%), which did not harbor *JCV*. This observation seems obvious if we take into account that colon cancers that arise in the context of hereditary nonpolyposis colon cancer (HNPCC) are often poorly differentiated (Jass, 2007; Alexander *et al*., Kim *et al*., 1994 2001). Those results were consistent with what we found in the current study.

Also, our results agreement with Feryel Ksiaa *et al.*,(2015) who was found that *JCV* status was significantly correlated with tumor differentiation (p=0.03).

Nosho *et al*.,(2009)have demonstrated that *JCV* T antigen expression was inversely associated with proximal location, high grade, and mucinous component. These results are disagreement with current results in this study.

Rehab *et al.*,(2013) who found the glandular *JCV* expression was significantly associated with high grade (p = 0.03), high mitotic index (p=0.02) and low apoptotic index (p = 0.00). These results are incompatible with our results.

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