Molecular and Immunohistochemical Detection of JC Polyomavirus in Human Colorectal Cancer in Sample of Iraqi Patients

Dalya Basil Hana M.Sc., College of Pharmacy/ University of Mustansiriyah, Assistant Professor
Dr.Azhar A.F.Al-Attraqhchi, College of Medicine/Al-Nahrain University, Professor Dr.Yaarub I. Khattab, College of Medicine/Al-Nahrain University.

Abstract : Background: Colorectal cancer initiates in the epithelial cells lining the colon and rectum. Since 1980, there has been a dr amatic increase in research on infections and cancer. There is mounting evidepnce that John Cunningham Virus (JCV) may be associated with several human cancers even in the absence of immunosuppression. JCV genomic sequences and oncogenic Large Tumor-antigen (LT-Ag) expression have been reported in a variety of human malignancies, including colon cancer.

Aim of the study: Determining the possible role of JCV in colorectal carcinogenesis by detection, quantification of JCV DNA load and demonstration of the Large Tumor antigen (LT-Ag) expression in both colorectal carcinoma tissues and normal colonic tissue biopsies.

Methods: From March/2013- April/2014, thirty tissue biopsies from patients with colorectal cancer (9 out of 60 tissue biopsies were taken from patients attended Al-Emamain Al-Khadhmyain City hospital, and 20 tissue colonic biopsies were taken from normal healthy individuals

Deoxyriboneucliec acid was extracted from all tissue biopsies that were enrolled in this study to detect and quantify JCV DNA by Real-Time PCR.

Tissue biopsies were kept in 10% buffered neutral formalin to prepare paraffin embedded blocks, which have been used in histopathological diagnosis and for Immunohistochemistry.

Statistical analysis performed with the statistical package for social sciences (SPSS) 19.0 and MicrosoftExcel 2013.

Results: Out of 30 colorectal carcinoma cases, 9(70.0%) were positive for detection and quantification of JCV in Real-Time PCR, compared to 4(20.0%) out of 20 normal colonic tissue biopsies. And 26(86.7%) were positive for LT-Ag expression in Immunohistochemistry, whereas non of the normal colonic tissues were positive.

Regarding viral load of JCV DNA, More JCV copies were present in colorectal carcinoma vs. normal tissue (mean 445.47 copies/µg

DNA vs. 101.50 copies/µg DNA, P<0.001).

Conclusion: Colorectal carcinomas contain more viral copies and express JCV T-Ag compared to colonic normal tissues. JCV and its LT-Ag oncogenic protein, may play a role in colorectal cancer development.

Key words: Colorectal cancer, JCV, LT-Ag.

Introduction: Colorectal cancer initiates in the epithelial cells lining the colon and rectum. The high rate replication of the epithelial cells of the human colon with 10^{10} epithelial cells being replaced every day is thought to contribute to the vulnerability of

colon and rectal epithelium to mutation and consequent carcinogenesis, although this elevated risk does not seem to apply to the small intestine despite comparably elevated cell turnover. If colonic epithelial cells accumulate mutations in oncogenes and tumor suppressor genes, the morphology of the cell changes, and there is a hyper proliferation of abnormal cells [1,2].

Since 1980, there has been a dramatic increase in research on infections and cancer. In 2002, it was reported that infectious agents either bacteria or viruses, accounted for ~18% of all cancers worldwide and infectious disease considered as an important pathogenic elements in human cancer, especially in the gastrointestinal tract [3].

There is mounting evidence that John Cunningham Virus (JCV) may be associated with several human cancers even in the absence of immunosuppression. JCV genomic sequences and oncogenic Tumor-antigen (T-Ag) expression have been reported in a variety of human malignancies, including colon cancer [4-7].

Some studies to assess the role of JCV in human colon cancer, have found that JCV T Ag DNA sequences are expressed in neoplastic tissues of the colonic mucosa but sequences were excluded from adjacent nonneoplastic colonic epithelial tissues [4,8].

The detection of viral proteins, including T-antigen, in the tumor cells indicate the potential involvement of JCV in pathways leading to the development and or progression of cancer.

Materials and Methods:

All samples were taken from patients who attended the endoscopic unit of Al-Emamain Al-Khadhmyain Hospital and Gastroentrology and Liver Center at Baghdad Teaching Hospital during the period (from March/2013 to April/2014) including 30 tissue biopsies from patients with colorectal cancer (9 out of 60 tissue biopsies were taken from patients who clinically diagnosed with colorectal carcinoma attending Al-Emamain Al-Khadhmyain City hospital for elective surgical colectomy), and 20 tissue colonic biopsies were taken from normal healthy individuals (normal colonoscopy) as a control group.

After histopathological diagnosis the collected tissue biopsies were diagnosed with colorectal adenocarcinoma. Tissue biopsies were collected from 50 patients, 25 were males and 25 were females.

Data were collected through direct interview with the patient, exclusion criteria were applied on those patients with colorectal carcinoma, who were underwent previous colectomy, chemotherapy intake, case history for colorectal carcinoma, malignancy tumor at another site, and family history for colorectal carcinoma.

Sample collection:

Four punch colonic tissue biopsies were taken from each patients enrolled in this study. Two punch biopsies were kept in a sterile tube of normal saline and preserved at -80 º C, which have been used in molecular tests, and two other biopsies were kept in 10% buffered neutral formalin to prepare paraffin embedded blocks, which have been used in histopathological diagnosis and for Immunohistochemistry.

Molecular Detection of JCV in Colorectal carcinoma:

Deoxyribonucleic Acid (DNA) was extracted from all tissue samples that were collected and kept in normal saline during this study by using of QIAamp DNA min Kit (Qiagene-Germany).

JCV was detected and quantified in Mx3005P Real-Time PCR system Stratagene (USA) by using of Quantification of Polyomavirus JC (Non coding regulatory region)-TaqMan® based JCV Advanced kit (Path-JCV), and Qasig^{TM}2×qPCR MasterMix Primer Design (UK) [9].

Histopathological and Immunohistochemical Study:

Two sections of 6 micrometers thickness were taken from each paraffin embedded tissue block. First sections were put on ordinary slide for Hematoxylin and Eosin (H&E) staining to confirm diagnosis and to detect the type of carcinoma and its histological grade.while the second sections were put on the charged slide for Immunohistochemistry (IHC) using EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436) to detect the expression of monoclonal antibody of Large T antigen of SV40 that cross react with this antigen.

Immunohistochemical Scoring:
The cellular immunoreactions of the tissue samples were scored quantitatively, and were classified into four groups according to the percentage of tumor cell nuclei that stained:
− indicates negative immunoreactivity
+ indicates 1–30% cell positivity
++ indicates 31–60% cell positivity
+++ indicates > 61% cell positivity [6].

Statistical Analysis:

Statistical analysis of this prospective study performed with the statistical package for social sciences (SPSS) 19.0 and MicrosoftExcel 2013. Categorical data formulated as count and percentage. Chi-square test used to describe the association of these data. Alternatively, Fisher exact test was used if there is 25% of cells less than expected count. Numerical data were described as mean, standard error of mean and standard deviation. Independent sample t-test used for comparison between two groups.

Relative risk (RR) is the ratio of the probability of an event occurring in an exposed group to the probability of the event occurring in a comparison, non-exposed group.The lower level of accepted statistical significant difference is bellow or equal to 0.05.

Results
Clinicopathological Findings:
Age: The mean age of patients with colorectal carcinoma was (60.30$\pm$12.51) years, with a range of 25 to 83 years. The mean age of normal colonic (control) group was (44.05$\pm$16.72) years, ranged from 19 to 73 years. The data showed that there was a

significant difference in the mean age of carcinoma group in compared to control groups (P<0.001) (table 1).

Table.1: The mean age of studied groups.

Age (years)	*Mean±Std. Deviation*	*Std. Error*	*Minimum*	*Maximum*
Carcinoma	63.30±12.51	2.28	25.00	83.00
Control	44.05±16.72	3.74	19.00	73.00

Gender: Out of 30 colorectal carcinoma patients, 15(50.0%) patients were females and 15 (50.0%) were males. Out of 20 control cases, 10 (50.0%) patients were females and 10 (50.0%) were males. There was no significant difference in gender distribution between the studied groups (table 2).

Table.2: Distribution of Carcinoma and Control Groups According to Gender.

Study groups		**Gender type**		**Total**
		Female	***Male***	
Carcinoma	Count	15	15	30
	%	50.0%	50.0%	100.0%
Control	Count	10	10	20
	%	50.0%	50.0%	100.0%

P value = 0.079

Tumor Site: A number of 17 (56.7%) cases of carcinoma were located in the left colon (descending and sigmoid colon) and 10 (33.30%) cases were on the right side (ascending colon), while 3 (10.0%) were located in rectum. (table 3).

Table.3: Distribution of Carcinoma Cases According to Site

Study groups		**Site**			Total
		Left	**Right**	**Rectum**	
Carcinoma	Count	17	10	3	30
	%	56.7%	33.3%	10.0%	100.0%
Control	Count	9	6	5	20
	%	45.0%	30.0%	25.0%	100.0%

P value = 0.181

Histopathological types of colorectal carcinomas: Carcinoma cases were divided into 27 cases (90%) non-mucinous and 3 (10.0 %) mucinous (figure 1).

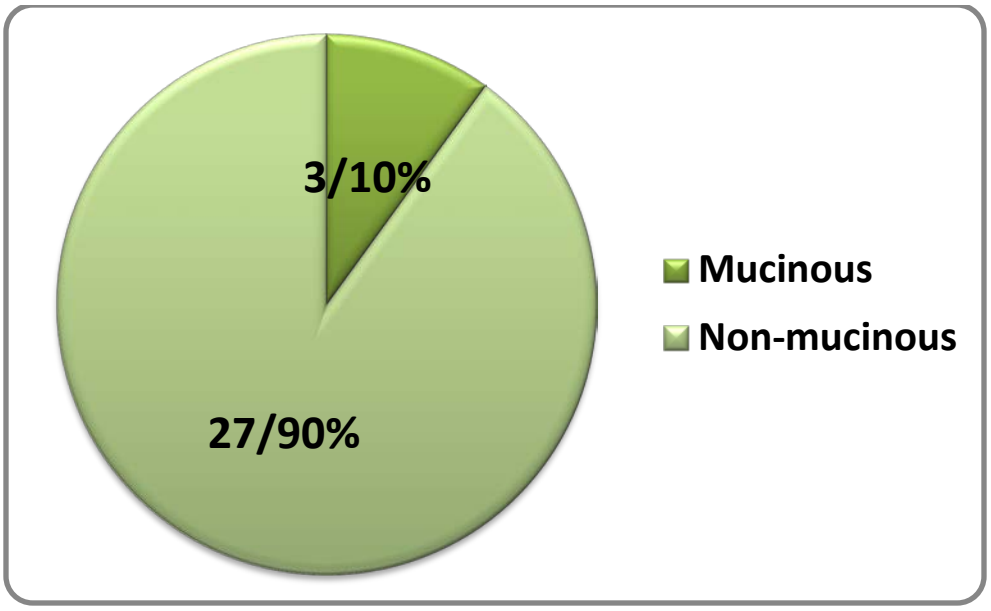

Figure. 1: Histopathological distribution of colorectal carcinoma.

Histological Grading: Most of the cases of colorectal carcinomas were moderately differentiated, 27 out of 30 (90%). Three (10.0%) cases were poorly differentiated (figure 2).

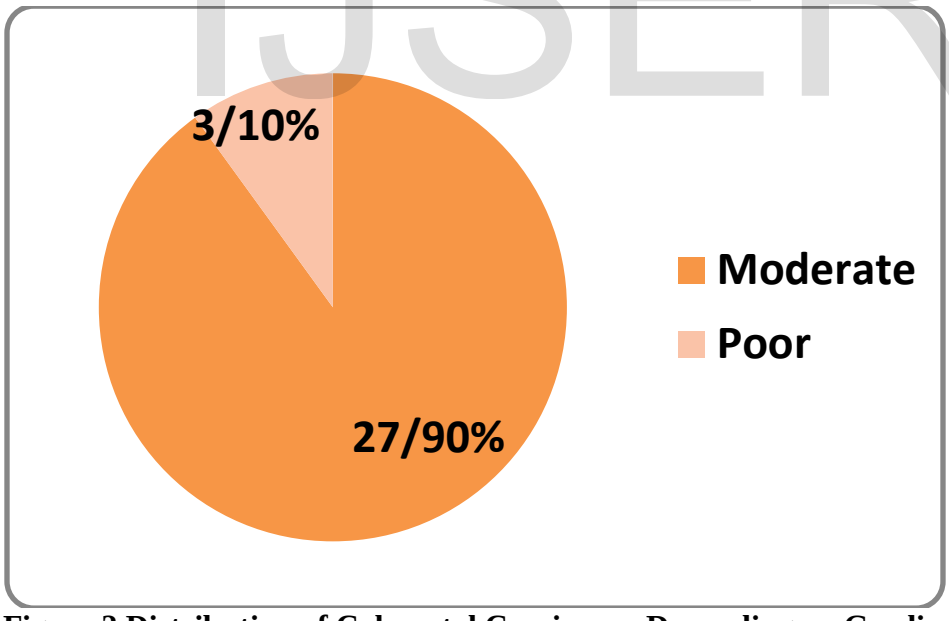

Figure.2 Distribution of Colorectal Carcinoma Depending on Grading.

Molecular Detection of John Cunningham Virus in Colorectal Carcinoma and Normal Colonic Tissue Biopsies: The concentration and the purity of extracted DNA samples were measured by Nanodrop, and the samples of a purity ranged from 1.7 to 1.9

were enrolled for the molecular detection of JCV in colorectal carcinoma and normal colonic tissue biopsies (control group) in this study.

Detection and Quantification of JC Virus: Results showed that JCV was detected in 9 (30%) out of 30 cases of the colorectal carcinoma, and 4 (20%) out of 20 normal colonic tissue biopsies (control group). There was no significant difference concerning the presence of JCV between the studied groups of the current study table (4. 5). The Relative Risk (RR) at 95% Confidence Interval (CI) of JCV detection in colorectal carcinoma was (1.5). All of these results suggested that the presence of JCV in colon and rectum may increase the risk for cancer development (table.4).

Table.4: Distribution of JCV Among Studied Groups and the Relative Risk for Colorectal Carcinoma Development

Study groups		JCV Real-Time PCR		Total	***RR (CI)***	***P value***
		Negative	Positive			
Colorectal Carcinoma	Count	21	9	30	***1.5 (0.53-4.21)***	***0.429***
	%	70.0%	30.0%	100.0%		
Control	Count	16	4	20		
	%	80.0%	20.0%	100.0%		

It was also showed that the viral load of JCV in colorectal carcinoma cases was ranged (290.28 - 690.34 copies/µg), and in normal colonic tissue and biopsies (control group) was (63.71 – 137.48 copies/µg). In addition the mean load of JCV was (445.47±139.16) in colorectal carcinoma tissue and biopsies, while it was (101.50±36.80) in normal colonic tissue biopsies. The results showed that the viral load of JCV was significantly different between colorectal carcinoma cases and normal colonic cases, P value < 0.001(table.5).

Table.5: Viral load of JCV (copies/µg) among colorectal carcinoma and control group

JCV-viral load	Colorectal Carcinoma	Control
Mean	445.47	101.50
SD	139.16	36.80
Median	403.11	102.41
Percentile 25	338.83	70.13
Percentile 75	560.44	132.88

P value < 0.001

Detection of JCV DNA according to different clinicopathological parameters in colorectal carcinomas

There was no significant in the detection of JCV DNA by Real-Time PCR between carcinomas and normal colonic group according to age, gender, tumor site, and tumor grade (table.6).

Table.6: Detection of JCV DNA According to Clinicopathological Parameters in Colorectal Carcinomas and Normal Colonic tissues

Clinical Pathology		Carcinoma		P value
		Negative	Positive	
Age groups	<50 years	2	1	1.000
	%	9.5%	11.1%	
	=>50 years	19	8	
	%	90.5%	88.9%	
Gender type	Female	11	4	1.000
	%	52.4%	44.4%	
	Male	10	5	
	%	47.6%	55.6%	
Site	Ascending	3	1	0.623
	%	14.3%	11.1%	
	Transvers	2	3	
	%	9.5%	33.3%	
	Descending	6	2	
	%	28.6%	22.2%	
	Sigmoid	6	2	
	%	28.6%	22.2%	
	Rectum	4	1	
	%	19.0%	11.1%	
Histological type	Mucinous	0	3	0.020
	%	0.0%	33.3%	
	Non-mucinous	21	6	
	%	100.0%	66.7%	
Tumor grade				0.535
	Moderate	18	9	
	%	85.7%	100.0%	
	Poor	3	0	
	%	14.3%	0.0%	

Immunohistochemical study: The mean expression of LT-Ag in carcinoma was (65.33±26.75), while there was no expression of LT-Ag in all normal colorectal tissue (control cases group). LT-Ag expression was significantly higher in carcinoma than in control cases P value = <0.001, (table.7).

Table.7: Immunohistochemical Expression of LT-Ag in Colorectal Carcinoma, and Control Group

	IHC Expression			
Study Groups	Mean	Std. Deviation	Std. Error	P value
Colorectal carcinoma	65.33	26.75	4.88	<0.001
Control	0.00	0.00	0.00	

Immunohistochemical expression of LT-Ag of JCV according to different clinicopathological parameters in colorectal carcinomas

There was no significant difference between colorectal adenoma group and control group according to clinicopathological parameters, age, gender, tumor site, histological type and tumor grade (table.8).

Gender: There was no significant difference of LT-Ag expression between males and females in all cases of this study P value = 0.701(table.8).

Tumor site: There was no significant difference in expression of LT-Ag according to tumor site P value = 0.326 (table.8).

Histological type: LT-Ag was expressed in all mucinous carcinoma included in this study (100%). There was no significant difference between mucinous and non-mucinous carcinoma P value = 0.640 (table.8).

Tumor Grade: The expression of LT-Ag in all cases of carcinoma was not significant, P value = 0.3 (table.8).

Table.8: Immunohistochemical expression of LT-Ag of JCV according to different clinicopathological parameters in colorectal carcinomas

Colorectal Carcinoma			**IHC**		Total	p value
			Negative	Positive		
Gender type	Female	Count	2	13	15	0.701
		%	13.3%	86.7%	100.0%	
	Male	Count	2	13	15	
		%	13.3%	86.7%	100.0%	
Age groups	<50 years	Count	0	3	3	0.640
		%	0.0%	100.0%	100.0%	
	=>50	Count	4	23	27	

	years	%	14.8%	85.2%	100.0%	
Site	Left	Count	1	16	17	0.326
		%	5.9%	94.1%	100.0%	
	Right	Count	2	8	10	
		%	20.0%	80.0%	100.0%	
	Rectum	Count	1	2	3	
		%	33.3%	66.7%	100.0%	
Histopathological type	Mucinous	Count	0	3	3	0.640
		%	0.0%	100.0%	100.0%	
	Non-mucinous	Count	4	23	27	
		%	14.8%	85.2%	100.0%	
Tumor grade	Moderate	Count	3	24	27	0.360
		%	11.1%	88.9%	100.0%	
	Poor	Count	1	2	3	
		%	33.3%	66.7%	100.0%	

Staining Scoring:

Scoring of expression of LT-Antigen of JCV in the current study classified into (-, +, ++ and +++) according to the number of cell positive and its association with the study groups (carcinoma and control). Brown nuclear staining was considered positive reaction and compared with the nuclear staining of the positive control slide that used in this study (figure.3).

Classification of carcinoma, adenoma and control groups into different grades of scoring (-, +, ++ and +++) showed significant difference ($p<0.001$). LT-Antigen staining with score +++ was mainly seen in carcinoma cases 25 out of 30 (83%), While no (0.0%) expression for LT-antigen was seen in control group, (table 9.) and (figure.4).

Table.9: Staining Scoring of LT-Ag of JCV in Colorectal Carcinomas

P value <0.001		Colorectal carcinoma N (%)	Control N (%)
IHC score	-	4 (13.3%)	20 (100%)
	+	0 (0%)	0 (0%)
	++	1 (3.3%)	0 (0%)
	+++	25 (83.3%)	0 (0%)
Total		30 (100%)	20 (100%)

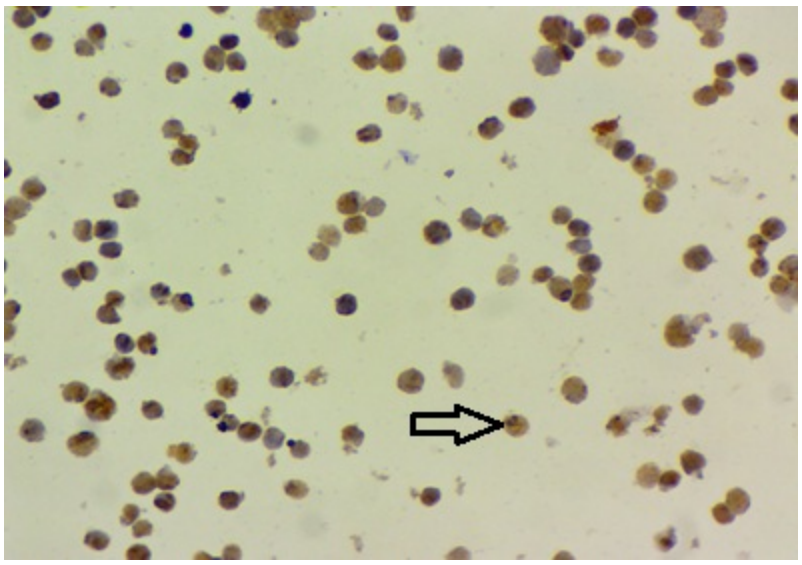

Figure .3: Excessive shedding reactive atypical mesothelial cell with rare mature lymphocytic cells (40X).

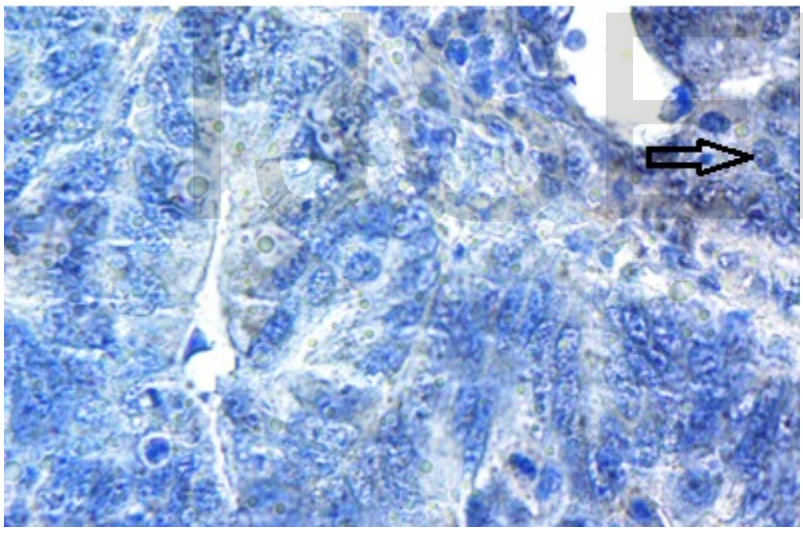

Figure .4: Moderately differentiated non mucinous colonic adenocarcinoma with complex infiltrating glandular architectural pattern showing positive LT-Antigen expression (40X).

Discussion:

Clinicopathological parameters of colorectal carcinomas

Age: Age is the most important risk factors for developing of colorectal cancer. The peaks of the occurrence of colorectal carcinoma in this current study is at about 80 years and more, with a mean age of (60.30±12.51) years, this is compatible other Iraqi studies done by Qasim BJ, Al –Tamimi , and Qasim ZJ who observed that the mean age of

patients with colorectal carcinoma was (56.03±2.37), 52.34, and was (56.88+1.99 years) respectively [10,11,12].

Gender: In the present study male patients comprised 50.0% of the total cases of colorectal cancer and 50.0% of the cases were females. This observation is disagree with other literatures which cited that the age-adjusted incidence of colorectal cancer for men exceeds that for women done by Fenoglio-Preiser *et al.* and American cancer society [13,14].

Tumor site: In this study, colorectal carcinomas were more frequent in the left side of colon (56.7 %) than the right side (33.3 %) with only (10.0 %) in the rectum.

Histopathological Types and Grade of Colorectal Carcinomas: Most colorectal carcinoma cases were non-mucinous in type which approximately agrees with previous studies in Iraq by Al –Sammak and, Qasim ZJ[15,4].

In the current study, most of the cases of CRC were moderately differentiated which is in accordance with other Iraqi studies done by Al-Sammak, Al -Tamimi, and Qasim who displayed that most of their colorectal carcinomas were moderately differentiated [15,3,4]. From another countries, an Egyptian study done by Rehab *et al.*, showed that (49%) of colorectal carcinomas were moderately differentiated [16].

Molecular Detection of John Cunningham Virus in Colorectal Carcinoma and Normal Colonic Tissue Biopsies: The results in this study showed that JCV was present in 9 (30%) samples of colorectal carcinoma. Regarding the quantity of JCV (viral load), the results showed that the viral load of JCV in colorectal carcinoma cases was ranged (290.28 - 690.34 copies/µg), while in normal colonic tissue biopsies (control group) were (63.71 – 137.48 copies/µg).

And the results showed that there was a significant difference of viral load between colorectal carcinoma and normal colonic cases.

Hit and run hypothesis explaining how some viruses might cause cancer and then mysteriously disappear, by the ‘‘hit-and-run’’ mechanism, where JCV infection contributed at an early stage to oncogenic progression e.g. by chromosomal instability, but JCV is no longer detectable after full progression to malignancy when diagnosis is made [17].

In this mechanism the viral DNA had been lost from the tumors but the tumors brought all the hallmarks of a virus having once been there [18].

Thus depending on this mechanism of JCV life cycle inside the cell, we can explain or suppose why the virus disappeared from the tissue during the time of sample collection or it can’t be detected by Real-Time PCR.

The results of the current study showed that JCV was detected in 4 (20.0%) with viral load range (63.71 – 137.48 copies/µg of DNA) by Real-Time PCR technique, while 16 (80.0%) of the samples didn’t show presence of JCV DNA. And this was agreed with a previous study that showed the viral load was 100-fold lower in the adjacent colonic epithelium 50–450 viral copies/µg of DNA [19].

Enteric cells normally do not support JCV replication but it can be speculated that JCV might enter such cells. The virus would not replicate but as basal cells are dividing viral genomes might be reproduced in a mechanistic way, altogether with the cellular genome [9]. The results of this study showed that the presence of low number of viral genomes in normal mucosa samples are compatible with such speculation.

Immunohistochemical expression of Large Tumor Antigen (LT-Ag) of JCV in carcinomas and normal colorectal tissues.

The result of this study is compatible to the literatures by P.Y. Lin et al., Goel et al., which have studied the expression pattern of JCV T-antigen in both colorectal neoplastic and normal mucosa [20,21].

Most of the JCV carcinogenic potential is attributed to JCV oncoprotein LT-Ag, which directly interacts with tumor suppressors and cell-cycle regulators to disrupt the cell cycle and prevent apoptosis. This oncoprotein is a viral replication protein, which has an important role in the life cycle of the virus by directing the infected cell into a physiological state supportive of virus gene expression and replication, as the virus does not encode enzymes necessary for DNA replication [22].

Regarding the size of the sample in this study, it is possible that the frequencyof T-Ag expression would be higher if we had studied more advanced carcinomas, because a majority of the cases in our study were obtained from biopsies rather than colectomy, and provided us with a relatively small area to examine for T-Ag expression.

Our finding that LT-Ag is expressed and detection of JCV DNA in colorectal carcinomas and is relevant because the presence of JCV DNA alone does not prove an active biological role for this polyomavirus. Furthermore, JCV DNA are frequently present even in normal, healthy individuals, and the demonstration of LT-Ag expression in colorectal carcinomas and with its presence exclusively in the nuclei of cells, and its absence from normal cells provides additional evidence that the JCV may play an important role in the early stages of colorectal carcinogenesis.

Conclusions and recommendations: Molecular detection of JCV genome and, more importantly, expression of its proteins in tumor cells suggests a role as a cofactor, in the development of colorectal cancer. However, further research is necessary which include a large number of samples in correlation with other tumor markers related to JCV infection.

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