



## Original Research Article

# Molecular and Immunohistochemical Detection of JC Polyomavirus in Human Colorectal Polyps in sample of Iraqi Patients

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## ABSTRACT

JC virus (JCV) is a member of polyomaviruses which may be associated in the pathogenesis of colorectal cancer, however, its role in premalignant lesions is unknown. The hypothesis that JCV DNA sequences and T-antigen (T-Ag) expression may be present in adenomatous polyps of the colon was tested. Determining the possible role of JCV in colorectal polyps by detection, quantification of JCV DNA load and demonstration of the Large Tumor antigen (LT-Ag) expression in both colorectal adenoma tissues and normal colonic tissue biopsies. From March/2013- April/2014, thirty tissue biopsies from patients with colorectal polyps, the tissue biopsies were taken from patients attended Al-Emamain Al-Khadhmyain City hospital, and 20 tissue colonic biopsies were taken from normal healthy individuals. Deoxyriboneucleic 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. Out of 30 colorectal adenoma cases, 8 (26.7%) 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 20(66.7%) 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 155.49 copies/ $\mu$ g DNA vs. 101.50 copies/ $\mu$ g DNA,  $P < 0.001$ ). Colorectal adnomas 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 tumor development.

### Keywords

JC Polyomavirus, Molecular and Immunohistochemical Detection, JCV DNA sequences and T-antigen (T-Ag)

## Introduction

Colorectal polyps are small growth of tissue into the lumen of the bowel, without

implying pathological relevance. Polyps can be stalked, round, or sessile and can vary in

size. They can occur as solitary or multiple polyps. Polypoid lesions are the most common pathological finding of colonoscopy. Polyps can only be classified by histological evaluation. Some larger polyps can eventually develop into cancer, while most diverticular polyps will not develop into colon cancer, most internal colon polyps will. Epidemiologic studies have shown that the growing of adenoma may reflect an innate or acquired tendency of the colon to form tumors, or adenomas are the primary precursor lesion of colon cancer [1,2].

Colorectal cancer develops through a stepwise progression of multiple genetic and epigenetic alterations, which manifest the transition from normal colonic mucosa to adenocarcinomas via adenomas (or adenomatous polyps) of the colorectum [3].

John Cunningham virus (JCV) is a member of the genus Polyomavirus from the Polyomaviridae family. The name polyoma (poly, a Greek word) means (many), and oma (tumors), and refers to the capacity of these viruses to cause tumors [4].

## **Materials and Methods**

All samples were taken from patients who attended the endoscopic unit of Al-Emamain Al-Khadhmyain City Hospital and Gastroenterology (from March/2013 to April/2014). Thirty colonic tissue biopsies were taken from patients with colorectal polyps and 20 tissue colonic biopsies were taken from normal individuals (normal colonoscopy) as a control group.

After histopathological diagnosis the collected tissue biopsies were diagnosed with colorectal adenomas. Tissue biopsies were collected from 50 patients, 20 were males and 10 were females.

## **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 ° 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 QasigTM2×qPCR MasterMix Primer Design (UK) [5].

## **Histopathological and Immuno-histochemical 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 adenoma and its histological types.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.

### **Statistical Analysis**

Statistical analysis of this prospective study performed with the statistical package for social sciences (SPSS) 19.0 and Microsoft Excel 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 below or equal to 0.05.

## **Result and Discussion**

### **Age**

The mean age of patients with colorectal adenoma was (44.23+16.56) years, ranging from 17 to 79 years. The mean age of normal colonic (control) group was

(44.05+16.72) years, ranged from 19 to 73 years (table .1). There is no significant difference in the mean age of adenoma in comparison with control group (P=0.967).

Gender: Out of 33 colorectal adenoma patients, 10 (33.3%) patients were females and 20(66.7%) were males, and out of 20 control cases, 10 (50.0%) were females and 10 (50.0%) were males. There was no significant difference in gender distribution among the studied groups, P value = 0.347, (table 4.2).

Tumor site: There was 1 (3.3%) case located in the ascending colon, 3 (10.0%) cases located in transverse colon, 6 (20.0%) were located in descending colon, 11 (36.6%) were in sigmoid colon, and 9 (30.0%) were located in the rectum.

Histopathological types of colorectal adenomas: Twenty four (80%) out of 30 colorectal adenoma were tubular, 5 (17%) were tubulovillous, and only one (3%) was villous adenoma, (figures.1).

Dysplasia in colorectal adenomas: Out of 30 cases of adenoma, 20 (67%) ones showed mild dysplasia, and only 10 (33 %) cases were with moderate, (figures.2).

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 8 (26.7%) out of 30 cases of the colorectal

adenoma, 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 adenoma was (1.5) (table.3).

It was also showed that the viral load of JCV in colorectal adenoma cases was ranged (59.67 – 309.71 copies/ $\mu$ g), and in normal colonic tissue and biopsies (control group) was (63.71 – 137.48 copies/ $\mu$ g). In addition the mean load of JCV was (155.49 $\pm$ 89.61) in colorectal adenoma tissue and biopsies, while it was (101.50 $\pm$ 36.80) in normal colonic tissue biopsies. The results showed that there is no significant difference between colorectal adenoma and normal colonic cases, \* P value = 0.431 (table.4).

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

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 dysplasia (table.5).

Immunohistochemical study: The mean expression of LT-Ag in adenoma was (25.33 $\pm$ 20.47), while there was no expression of LT-Ag in all normal colorectal tissue (control cases group). LT-Ag expression was significantly higher in adenoma than in control cases P value =  $\square$ 0.001, (table.6).

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 dysplasia (table.7).

Staining Scoring: 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 adenoma and control groups into different grades of scoring (-, +, ++ and +++) showed significant difference (p<0.001). LT-Antigen staining with score ++ that was mainly seen in adenoma cases 12 (40%) out of 30, While no (0.0%) expression for LT-antigen was seen in control group, (table .8) and (figure.4).

#### **Clinicopathological parameters of colorectal adenomas**

Age: Age is of the most important risk factors for developing of colorectal tumor. Dragovich and Tsikitis suggested that the timeline for progression from early premalignant lesion to cancer ranges from 10-20 years [7].

The frequency of cases of colorectal adenoma in this study increases with age which was also demonstrated by Ramji and Yoshida who showed that the prevalence and distribution of adenoma increases with age [8].

And the frequency peaks was at the age of 70 years and more, this is agree with Fenoglio-Preiser et al. who reported that adenoma incidence peaks at age 60 or 70 years [9]and also agree with an Iraqi study bone by Nussrat who observed that colorectal adenomas were detected mainly in the age group 50-69 years [10].

**Gender:** In the present study gender distribution in colorectal adenoma, males exceeds females with 66.7% of cases being males and 33.3% of cases were females, this result was in accordance with an Iraqi study done by Nussrat, showed that the gender distribution of colorectal adenoma was 60% in male compared to 40% in female [10]. The results of this study is disagree with an Egyptian study done by Ahmed et al., who they showed that there was no statistically significant difference regarding the distribution of polyps according to gender, they showed that colorectal polyps were present in (9.4%) of males and in (9.8%) of females [11].

**Tumor site:** In the current work, colorectal adenomas were more frequent in the left colon (60.0 %) than the right colon (30.0 %) with (10.0 %) occurrence in the rectum.

Rosai recorded that adenomatous polyps are distributed rather regularly throughout the large bowel, with 40% found in the right colon, 40% in the left colon, and 20% in the rectum [12]. In Iraq, Nussrat showed that more than half of cases of colorectal adenomas were found in the left colon (53.1%) and (29.7%) were found in the right colon and 17% of the tumors were rectal [10]. These results are in accordance with that of the present study.

**Histopathological types and dysplasia in colorectal adenomas:** Concerning the microscopical types of colorectal adenoma, tubular adenoma was the predominant type (80.0%), followed by tubulovillous (16.66%) while villous adenoma was the least one (3.3%). This distribution is agree with that obtained by Fenoglio-Preiser et al. and with study done by Abdulkader et al., who proved that tubular adenoma is the most common type, accounting for (68% to 87.1%) and (58.5%) of adenomas

respectively [9,13], and disagree with a study on Iraqi patients by Nussrat who said that (59.57%) of cases that included in his study were tubulovillous, (29.78%) were villous, and only (10.63%) of the cases were tubular adenoma [10]. This disagreement could be attributed to the environmental, racial and geographical differences, in addition to sample size difference.

When the degree of dysplasia is taken into consideration, 10 (33.33%) out of 30 cases were showed mild dysplasia and the rest of cases 20 (66.67%) were moderately dysplastic. These findings go with that obtained by M. Ifrim, C. Toth, and C. Precup who displayed that (77.77%) of the cases have moderate dysplasia [14].

**Molecular detection of John Cunningham virus in colorectal adenoma and normal colonic tissue biopsies:**

Intestinal and, especially, colorectal diseases represent one of the most common medical problems of human beings, with colorectal carcinoma being one of the most frequently occurring malignancies through the world. There is many difficulties to determine the specific roles of biological agents that might be considered carcinogenic because of It's phenotypic complexity, and even more difficulties in determination of their causality, or implication at a particular stage in disease progression. Among the exogenous agents associated to cancer initiation or progression are some infectious agents, including viruses and bacteria [15].

Infectious diseases, on the other hand, do not represent mainstream oncologic research but their importance as pathogenic elements in human cancer increases [16].

JCV infects chronically the human gastrointestinal tract, through fecal



contamination, and remains there latently during the adulthood infecting probably without exceptions, all human populations [17,18].

Regarding the quantity of JCV (viral load), the results were compatible with similar quantitative viral loads in DNA from colorectal adenomas (9000 viral copies/ $\mu$ g of extracted DNA), while the viral load in the adjacent colonic epithelium was 100 fold lower (50–450 viral copies/ $\mu$ g of DNA) [19].

Because of the synthesis of JCV DNA is not regulated by the cell, it is more likely that changes in the viral genome or even integration into cellular genes would appear. Therefore low JCV copy number that diminishes or becomes undetectable as the malignancy develops is expected.

The presence of NCCR is essential for the proper replication of JCV, which may indicate that amplification failure or low NCCR (gene sequences that chosen to be amplified in this study) copy number might confer a prerequisite for incomplete or non-lytic course of the infection [10].

Hit and run hypothesis explaining how some viruses might cause cancer and then mysteriously disappear, 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 [20].

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, hit and run hypothesis, where the viruses can mediate cellular transformations through an initial hit, although maintenance

of the transformed state is compatible with the loss run of viral molecules [21,22].

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

There was a demonstration of JCV among the colorectal adenoma group and accordingly the results of this study suggested a possible role of JCV in the early stages of colorectal carcinogenesis.

In the control group, the results of the current study showed that JCV was detected in 4 (20.0%) with viral load range (63.71 – 137.48 copies/ $\mu$ g of DNA) by Real-Time PCR technique, while 16 (80.0%) of the samples didn't show presence of JCV DNA, this agree with a previous study done by George Theodoropoulos et al., that showed the viral load was 100-fold lower in the adjacent colonic epithelium (50–450 viral copies/ $\mu$ g of DNA) [19].

Previously it has been reported that the gastrointestinal tract might be an entry site for the virus [23]. Moreover, JC viral sequences have been described to be present in the enteric glia [24]. 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 [10].

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 colorectal adenomas was (66.7%). The expression of LT-Ag was concentrated in the nucleus of the cells. All control cases were negative for LT- Ag expression.

These results are compatible to that of literatures done by Enam et al., P.Y. Lin et al., Goel et al., Link et al., Nosho et al., Ogino et al., Selgrad et al. and Jung et al., which have studied the expression pattern of JCV T-antigen in both colorectal neoplastic and normal mucosa. About 35%-94% of CRC tissues and 5-50% of colorectal adenomas were found to host JCV LT-antigen, which is often concentrated in the nucleus [6,25,26,27]. The results of this study disagree with a study by Hori R et al., who failed to investigate LT-Ag in their samples [28].

The oncogenic potential of JCV was initially discovered when Walker et al. injected the virus into Syrian hamster brains and induced aneuploid tumors [29]. The oncogenic activity of its large T-antigen (LT-Ag) has raised the possibility that it can induce cancer development in human hosts. This was proved by Caracciolo et al. when his results revealed that the LT-Ag plays a critical role in host-cell life cycle as it directs the early and late viral gene expression, as well as the viral DNA replication during lytic infection [30]. However, based on inadequate evidence in humans and sufficient evidence in experimental animals, a WHO International Agency for Cancer Research Monograph

Working Group recently classified JCV as a possibly carcinogenic to humans [31].

In this study and regarding the size of the samples, it was shown that it is possible that the frequency of LT-Ag expression would be higher if the number of the samples will be increased and the more advanced adenomas cases will be enrolled in the study, because the majority of the cases were obtained from biopsies rather than polypectomies, and the area provided for examination was so small for T-Ag expression.

The findings of this study concluded that the LT-Ag is expression is an important factor for the development of each of adenomas while the presence of the viral DNA alone is not enough argument for the development of such diseases.

Furthermore, JCV DNA are frequently present even in normal, healthy individuals, and the demonstration of LT-Ag expression in colorectal adenomas with its presence exclusively in cells nuclei, and its absence from normal cells provides additional evidence that the JCV may play an important role in the early stages of colorectal tumorigenesis.

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 tumor. However, further research is necessary which include a large number of samples in correlation with other tumor markers related to JCV infection.

**Table.1** The mean age of studied groups

Age (years)	Mean+Std. Deviation	Std. Error	Minimum	Maximum
Colorectal adenoma	44.23+16.56	3.02	17.00	79.00
Control	44.05+16.72	3.74	19.00	73.00

**Table.2** Distribution of adenoma and control groups according to gender

Study groups		Gender type		Total
		Female	Male	
Adenoma	Count	10	20	30
	%	33.3%	66.7%	100.0%
Control	Count	10	10	20
	%	50.0%	50.0%	100.0%

P value = 0.347

**Table.3** Distribution of JCV among studied groups and the relative risk of colorectal adenoma

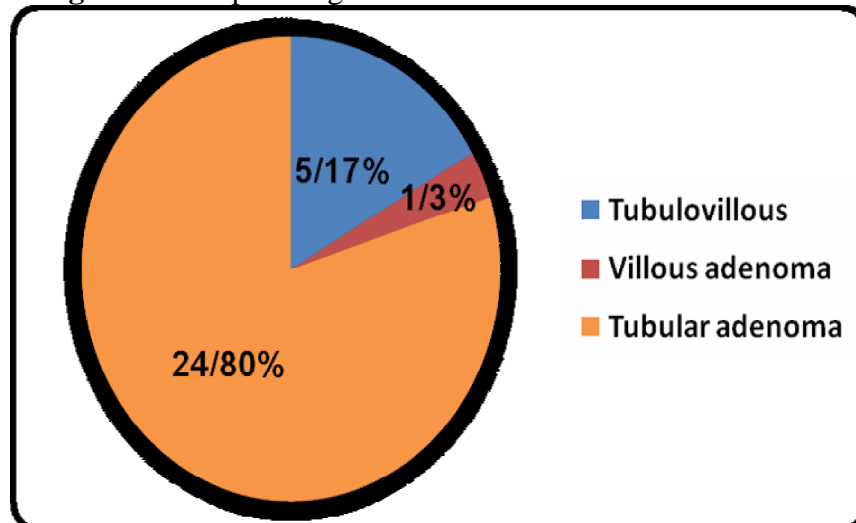
Study groups		JCV Real-Time PCR		Total	RR (CI)	P value
		Negative	positive			
Colorectal Adenoma	Count	22	8	30	1.33 (0.46-3.84)	0.588
	%	73.3%	26.7%	100.0%		
Control	Count	16	4	20		
	%	80.0%	20.0%	100.0%		

**Table.4** Viral load of JCV (copies/μg) colorectal adenoma and control group

JCV-viral load	Colorectal Adenoma	Control
Mean	155.49	101.50
SD	89.61	36.80
Median	123.16	102.41
Percentile 25	87.50	70.13
Percentile 75	226.63	132.88

\* P value = 0.431

**Figure.1** Histopathological distribution of colorectal adenoma





**Table.5** Detection of JCV DNA according to different clinicopathological parameters in colorectal adenomas

Clinical Pathology		Adenoma		P value
		Negative	Positive	
Age groups	<50 years	12	6	0.419
	%	54.5%	75.0%	
	=>50 years	10	2	
	%	45.5%	25.0%	
Gender type	Female	9	1	0.210
	%	40.9%	12.5%	
	Male	13	7	
	%	59.1%	87.5%	
Site	Ascending	1	0	0.143
	%	4.5%	0.0%	
	Transvers	2	1	
	%	9.1%	12.5%	
	Descending	6	0	
	%	27.3%	0.0%	
	Sigmoid	9	2	
	%	40.9%	25.0%	
Histological type	Rectum	4	5	0.163
	%	18.2%	62.5%	
	Tubulovillous	3	2	
	%	13.6%	25.0%	
	Villous Adenoma	0	1	
	%	0.0%	12.5%	
Dysplasia	Tubular adenoma	19	5	0.682
	%	86.4%	62.5%	
	Mild	14	6	
	%	63.6%	75.0%	
	Moderate	8	2	0.682
	%	36.4%	25.0%	

**Table.6** Immunohistochemical expression of LT-Ag in colorectal adenoma, and control group

Study Groups	IHC Expression			P value
	Mean	Std. Deviation	Std. Error	
Colorectal adenoma	25.33	20.47	3.74	<0.001
Control	0.00	0.00	0.00	

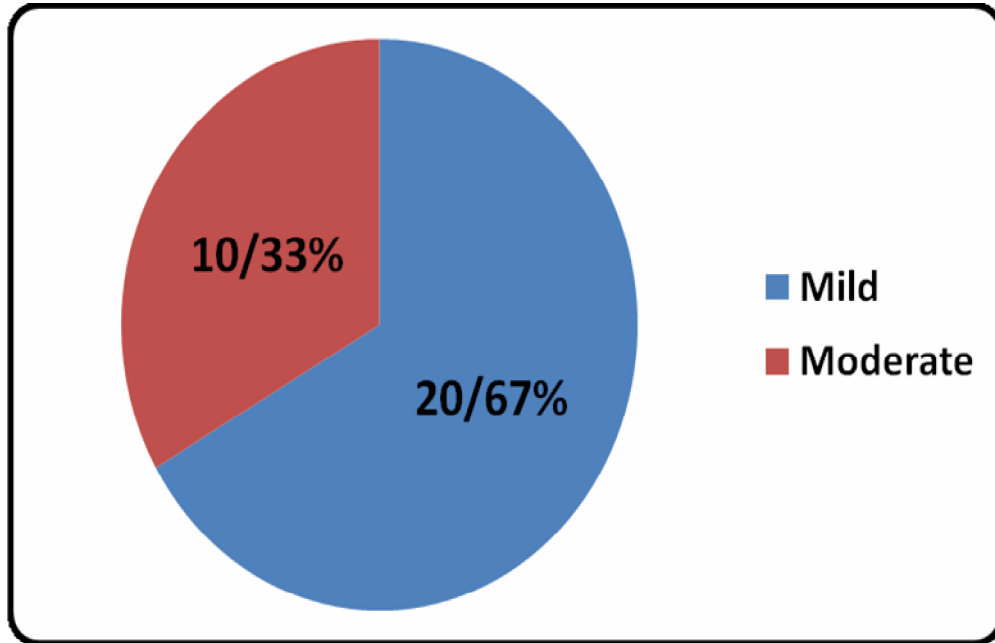
**Table.7** Immunohistochemical expression of LT-Ag of JCV according to different clinicopathological parameters in colorectal adenomas

Colorectal adenoma			IHC			p value
			Negative	Positive	Total	
Age groups	<50 years	Count	6	12	18	0.650
		%	33.3%	66.7%	100.0%	
	=>50 years	Count	4	8	12	
		%	33.3%	66.7%	100.0%	
Gender type	Female	Count	5	5	10	0.169
		%	50.0%	50.0%	100.0%	
	Male	Count	5	15	20	
		%	25.0%	75.0%	100.0%	
Site	Ascending	Count	1	0	1	0.309
		%	100.0%	0.0%	100.0%	
	Transverse	Count	1	2	3	
		%	33.3%	66.7%	100.0%	
	Descending	Count	3	3	6	
		%	50.0%	50.0%	100.0%	
	Sigmoid	Count	4	7	11	
		%	36.4%	63.6%	100.0%	
Rectum	Count	1	8	9		
	%	11.1%	88.9%	100.0%		
Histological type	Tubulovillous	Count	1	4	5	0.581
		%	20.0%	80.0%	100.0%	
	Villous Adenoma	Count	0	1	1	
		%	0.0%	100.0%	100.0%	
	Tubular adenoma	Count	9	15	24	
		%	37.5%	62.5%	100.0%	
Dysplasia	Mild	Count	8	12	20	0.251
		%	40.0%	60.0%	100.0%	
	Moderate	Count	2	8	10	
		%	20.0%	80.0%	100.0%	

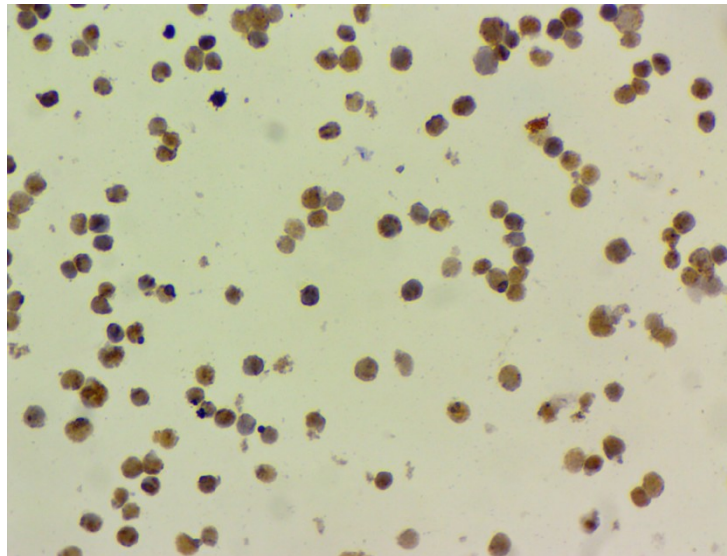
**Table.8** Staining scoring of LT-Ag of JCV in colorectal adenomas

		Study groups	
		Colorectal adenoma N (%)	Control N (%)
IHC score	-	10 (33.3%)	20 (100%)
	+	8 (26.7)	0 (0%)
	++	12 (40%)	0 (0%)
	+++	0 (0%)	0 (0%)
Total		30 (100%)	20 (100%)
		<0.001	-

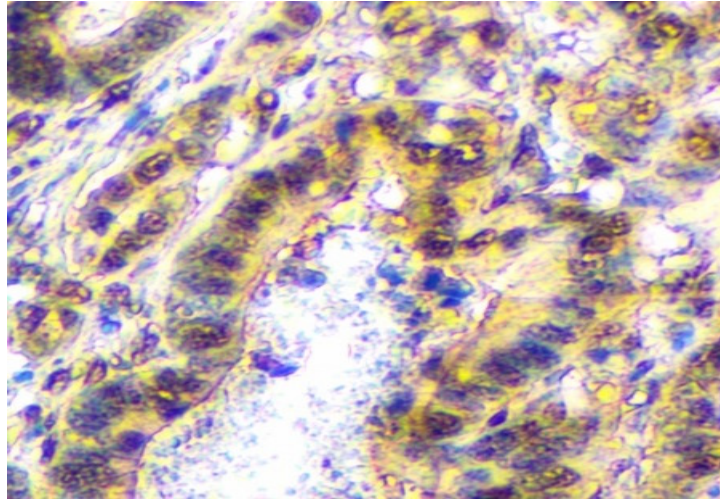
**Figure.2** Distribution of colorectal adenoma according to dysplasia



**Figure.3** Image of excessive shedding reactive atypical mesothelial cell with rare mature lymphocytic cells (40X)



**Figure.4** Image of a tubular adenomatous polyp lacking cytomorphological epithelial atypia, showing positive (arrows) LT-Antigen expression (40X)



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