

Original Research Article

The Validity of Real Time PCR and in Situ Hybridization in the Detection of JCV in Colonic Biopsies of Patient with Colorectal Cancer

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A B S T R A C T

John Cunningham virus (JCV) is a type of human polyomavirus. It's widespread virus detected in different population throughout the world. JCV encodes for T-Ag which have oncogenic capability through interaction with different regulatory pathways and can interfere with cell cycle control and genomic instability mechanisms. The association between JCV and colorectal carcinoma still awaits the final conformation. Determine the role of JCV in malignant transformation of colorectal carcinoma through the detection of T-Ag gene and agnoprotein gene and demonstrating JCV DNA in both colorectal carcinoma tissue and normal colonic tissue biopsies. involved the use of 28 colonic tissue biopsies taken through colonoscopy from patients with colorectal cancer and 31 tissue biopsies from patients without CRC attended to GIT Endoscopic Unit of Oncology Hospital, Baghdad Teaching Hospital, and Al-Yarmouk Teaching Hospital during the period from June 2013 to March 2014. JCV T Ag and agnoprotein were detected by real time PCR and in situ hybridization, Viral DNA load of positive samples were determined by quantitative real-time PCR. out of 28 CRC cases, 16(57.1%) were positive for T-Ag compared to 9/33(27.3%) in non CRC patient ($p=0.018$), 12/28(42.9%) of CRC cases were positive for agnoprotein gene compared to 5/33(15.2%) in non CRC patient ($p=0.016$). While 15/28(53.6%) of CRC were positive JCV DNA by ISH compared to 10/33(31.3%) in non CRC patient ($p=0.080$). Significant differences between the study groups regarding glandular involvement ($p=0.036$) signals intensity ($p=0.032$), and pattern of involvement while no significant differences were found regarding stromal involvement ($p=0.52$). JCV exists in colorectal cancer tissue more than normal tissue and may play a role as an etiology for colonic carcinogenesis.

Keywords

Colorectal cancer,
JCV,
LT-Ag

Introduction

The incidence of colorectal cancer in Iraq increased in comparison to a decreased incidence of stomach cancer. Colorectal carcinoma encompassed 2.6% of cancers paralleled to 6-13% in the developed countries while in the industrialized nations it represent 17-51.1% (Al-Humadi, and Buffalo 2008). This observation necessitates the need to look for new causative agents which may participate in causing the incidence of CRC.

John Cunningham virus (JCV) or (JCPyV) is a type of human polyomavirus (Padgett et al. 1971). JC virus is very common in the general population and it is detected in different population throughout the world, which varies from 44 - 90 percent of the human population (Walker et al. 1973) with 19-27% of these people shedding JCPyV in their urine (Bellizzi et al. 2013). JC virus is small encapsidated circular covalently linked double stranded DNA of 5.1 kilobases, supercoiled form, in icosahedral protein structure measuring 40nm in diameter (White et al. 2006). Six non-structural proteins are encoded in the viral DNA which includes large T antigen, small t antigen, T₁₃₅, T₁₃₆, T₁₆₅ and small regulatory protein agnoprotein. Large T antigen is named from its tumor promoting function (Khalili, Sariyer, and Safak 2008). The capsid is composed of three capsid proteins VP1, VP2, and VP3 with VP1 being the major constituent (Bollag et al. 2006).

There is increasing evidence that JCV may be associated with several human cancers even in the absence of immunosuppression. JCV genomic sequences and oncogenic T-Ag expression have been reported in a variety of human malignancies, including colon cancer (Laghi et al. 1999; Ricciardiello et al. 2001; Enam et al. 2002)

Patients and Methods

Sixty one patients attending the GIT endoscopic units of Oncology Hospital, Baghdad Teaching Hospital and Al-Yarmouk Teaching Hospital were selected as subjects in the period extended from June 2013 to March 2014. All study groups underwent diagnostic colonoscopic biopsies. Patient were further divided into 2 groups namely carcinoma group including 28 patients proved to have colorectal carcinoma their age ranged from 19-70 years and control group including 33 patients who did not have colorectal carcinoma, their age ranged from 20-70 years. Ethical approval for the study was obtained from the ethical committee of College of Medicine University of Baghdad. Two colonic biopsy sets were taken from each patient through colonoscopy. One set of the biopsies was fixed in 10% buffered neutral formalin to prepare paraffin embedded blocks, which have been used in histopathological diagnosis and for in situ hybridization. The second set of biopsies was kept in normal saline, immediately frozen at -20°C and submitted to molecular study.

Molecular Detection of JCV

The DNA was extracted from all tissue samples that were collected and kept in normal saline by using KAPA Express Extract kit (KAPA Biosystem, USA-KK7101) according to the manufacture instructions.

PCR amplification was performed by using two sets of primers previously used (Chapagain et al. 2006; Mou et al. 2012): JCT Ag-F (5'-AGA GTG TTG GGA TCC TGT GTT TT-3') and JCT Ag -R (5'-GAG AAG TGG GATGAA GAC CTG TTT-3') were used in the real-time PCR targeting a sequence of T-antigen gene while

JCV-AGNO-F-5'-GTC TGC TCA GTC AAA CCA CTG-3' and R-5'-GTT CTT CGC CAG CTG TCA C-3'(Enam et al. 2002; Wang et al. 2012) which amplify a region within coding region of JCV Agnoprotein.

Amplification was carried out on 20 ng of template DNA, 10ml of KAPA SYBR FAST qPCR Kit Master Mix (2X) Universal (KAPA Biosystem, USA) (Cat.No.KK4600) and 4 pmol each of forward and reverse primers. Thermal cycling was initiated with a first denaturation step at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 15 s and the amplification fluorescence were monitored at 60°C at the end of each cycle. A standard curve was constructed using serial dilutions (10^3 to 10^7 copies of JCV DNA) JCV Bio probe (ENZO life sciences, Ny. USA) were performed for every plate run starting with 1.81×10^7 copies / μ g with 10X dilution factor. Three replicates were performed for each sample and real-time PCR data were analyzed using Mx3005P Real-Time PCR system Stratagen (Agilent Technologies, USA).

Histopathological and in Situ Hybridization Study

Two sections of 4 micrometers thickness were taken from each paraffin embedded tissue block. First sections were put on ordinary slide for Haematoxylin and Eosin (H&E) staining to confirm diagnosis and to detect the histological types and grades of tumor.

The second sections were put on the charged slide for In situ hybridization (ISH) using Simply Sensitive Alkaline Phosphatase-NBT/BCIP In Situ Detection System (Cat. No.32870) from ENZO life Sciences (NY, USA). JCV BioProbe (Cat. No.-40847) was purchased from ENZO life Sciences (NY, USA).

Subsequent steps of deparaffinization and rehydration were performed in xylene and a decreasing concentration of alcohol respectively. The sections were deproteinized with proteinase K and incubated for 10 min at room temperature. JCV BioProbe was added. DNA was denaturated for 10 min at 95°C, and hybridization continued for 1 h at 37°C in humidified chamber. All the operations of visualization, staining and counterstaining were carried out according to the instructions of the commercial kit.

Positive reactivity was indicated by blue-purple deposits which vary in intensity from almost black (very strong) to faintly purple blue (weakly positive).

In situ hybridization was given intensity and percentage scores, based on intensity of positive signals and number of signals per 100 cells, respectively. The intensity score was classified in to: no signal, low intensity and high intensity. Positive cells were counted in 10 different fields of 100 cells for each sample and the range of positive cells of the ten fields was determined assigning cases to one of the two following percentage score categories: Score (0): No reaction, Score (+) = 1 – 5% and Score (++) = > 5 % (Casini et al. 2005; Samaka et al. 2012).

Statistical Analysis

Statistical analysis tests were applied to compare and correct results of different methodology applied in the present work. Frequencies of positive and negative results were recorded as percentages of the total. Chi square test with Fisher exact correction was applied to compare between frequencies. T-test was applied to compare between means. In each test, a p-value <0.05 was considered significant.

Cohen Kappa was used as an index of inter-rater reliability to measure agreement between 2 sets of dichotomous rating. A value of $K=0.4-0.59$ indicate moderate inter-rater reliability, $0.60-0.79$ indicate substantial and 0.8 indicating outstanding.

Results and Discussion

Age

The age of carcinoma group ranged from 19-70 years with mean (52.85 ± 11.74) years while the age of control group ranged from 22-73 years with mean age (46.75 ± 13.22) years. The results revealed no significant statistical difference in mean age between study groups. The highest frequency (35.71%) of patients with colorectal carcinoma was within 50-59 years. In the control group the highest frequency (33.33%) was within 50-59 years.

Gender

Out of 28 carcinoma group, 18 (64.28%) patients were males and 10 (35.72%) were females with male to female ratio (1.8:1). For the control group 26 (78.78%) were males and 7(21.22%) were females with male to female ratio (3.7:1). Individuals in carcinomas and control groups did not differ significantly according to sex ($P= 0.208$) (table 1).

Histopathological Types

Microscopic examination of H&E stained slides from carcinoma group showed that 24 out of 28 (85.72%) cases were of non-mucinous adenocarcinoma while only 4 (14.28%) cases were of mucinous adenocarcinoma (figure.2).

Histological grading of adenocarcinoma in carcinoma group showed that 21(75%) of cases was of moderately differentiated

adenocarcinoma while 3 (10.7%) cases were of well differentiated and only 4 (14.3%) cases were of poorly differentiated adenocarcinoma as shown in Figure (3)

Tumor Site

Thirteen out of 28 (46.42%) cases of the carcinoma group were from left colon, 7(25%) cases from right colon and 8 (28.58%) were from rectum. For control group 14 out of 33 (42.4%) were from left colon, 8 (24.2%) from right colon and 11 (33.3%) from rectum. Statistical analysis showed no significant difference ($P=0.919$) between study groups.

Detection and Quantification of JCV by Real Time PCR

Detection and Quantification of T-Ag Gene by Real Time PCR

The T-Ag gene was detected in 16 out of 28 (57.1%) in carcinoma group, while 9 out of 33 (27.3%) cases of the control group were positive for JCV T-Ag gene (Table 1). The results revealed significant difference between study groups ($P=0.018$). The viral load (VL) for carcinoma group ranged from $1.00e+02$ copies/ μ g to $8.39e+02$ copies/ μ g with a mean of (416.937 ± 217.779 SE 56.230) as shown in table (2). For control group the VL was ranged from to $9.88E+01$ to $4.11e+02$ copies/ μ g with a mean of (229.866 ± 111.492 SE 39.418). The difference between the two groups was statistically significant regarding the mean viral load; $p<0.05$.

T Ag gene detection by real time PCR was 57.14% sensitive and 72.73% specific. The positive predictive value (PPV) was 64% and the negative predictive value (NPV) was 66.67%.

Ten out of 21 (47.6%) cases of moderately

differentiated adenocarcinoma were positive for JCV T-Ag gene. Positive T-Ag gene was also detected in 2 out of 3 (66.7%) cases and 4 cases out of 4 (100%) in well and poorly differentiated adenocarcinomas respectively. However, the difference failed to reach the level of statistical significant ($p>0.05$).

Amplification plots of real time PCR for T Ag gene by real time PCR for carcinoma and control groups. Ct values ranged from 27.03 - 30.22 and 28.1-30.24 in carcinomas and control groups respectively. The photography was taken directly from the real time PCR machine Agilent technology.

Regarding Tumor Site

T-Ag gene was detected in 7(100%), 7 cases out of 13(53.85%) and only 2 out of 8 (25%) cases of right, left colon and rectum respectively in carcinomas group. While in control group T-Ag gene was detected in 1 out of 8 (12.5%) cases of Rt. colon, 5 out of 14 (35.7%) cases of LT colon and 3 out of 11 (27.27%) cases of rectum. A significant statistical difference was noticed in the carcinomas group, $p<0.05$.

Detection and Quantification of Agnoprotein Gene by Real Time PCR

The agnoprotein gene of JCV was detected in 12 out of 28(42.85%) cases of the carcinomas group while only 5 out of 33 (15.15%) cases of control group were positive (table 3). Statistical analysis of the results showed significant difference between study groups as ($p = 0.016$).

The viral loads of carcinomas group (table-3) were ranged from ($1.22e+02 - 4.55e+02$) copies/ μ g with a mean of (317 ± 129.121), while that of the control group were ranged from ($3.32e+02 - 6.45e+01$) copies/ μ g with a mean of (152.94 ± 105.149). The difference between carcinomas group and

control group was significant regarding the viral load ($P<0.05$).

The test showed a sensitivity of 42.86%, specificity of 84.85%, PPV of 70.59% and NPV of 63.64%.

Regarding grades of tumor, JCV agnoprotein gene was detected in 2 out of 3 (66.67%) cases, 7 out of 21(33.33%) and 3 out of 4 (75%) cases of well, moderately, and poorly differentiate adenocarcinoma respectively. No significant difference was found between the presence of JCV agnoprotein gene in the different grades of tumor among carcinomas group, $p = 0.206$.

Regarding site of tissue biopsies, Agnoprotein gene was detected in 6 out of 7(85.7%) cases, 5 cases out of 13(35.5%) cases and only 1 out of 8 (12.5%) cases of right, left colon and rectum respectively. A significant statistical difference was detected among the carcinomas groups ($P= 0.015$). While for control group only 1case out of 8 (12.5%) from RT colon and 4 cases out of 14(28.6%) from LT colon were positive for agnoprotein DNA. No significant difference was detected in the control group. Statistical analysis reached the level of significant difference on comparing variables between study groups ($p<0.05$).

Agreement and Inter-rater Reliability Between T-Ag Gene and Agnoprotein Gene in Study Groups

When comparing the results from T-Ag gene and agnoprotein gene by real time PCR, the results revealed that in carcinoma group both T-Ag gene and agnoprotein gene were detected in 12 cases out 28 (42.86%). On the other hand 12 out of 28 (42.86%) cases were negative for both T-Ag gene and agnoprotein gene. Only 4 out 28 (14.28%) cases were positive for T-Ag gene only. In the control group both T-Ag gene and agnoprotein gene were detected in 5 out of

33(15.15%) cases, and not detected in 24 out of 33(72.73%), while the rest 4 out of 33 (12.12%) cases showed the amplification for only T Ag gene. The agreement between the 2 tests was substantial for both carcinomas and control groups. The calculated kappa (K) for the carcinomas group was 0.72 and for the control group was 0.784.

Detection of JCV by CISH

The DNA genome of JCV was detected in tissue blocks of colorectal biopsies. JCV were detected in 15 out of 28 (53.57%) cases and in 10 out of 33 (30.33%) cases of carcinomas and control group respectively (table 5). No significant statistical difference was noticed between study groups ($p=0.080$). CISH technique for the detection of JCV DNA showed 53.57% sensitivity and 69.70% specificity. The PPV was 60% and NPV was 63.89%

Agreement and Inter-rater Reliability Between the Detection of JCV by qPCR and Detection of JCV by CISH

JCV T-Ag by qPCR and JCV by CISH

Fourteen out of 28 (50%) cases revealed positive for JCV DNA by qPCR for T-Ag and by CISH techniques, Eleven out of 28 (39.29%) cases were negative for both methods. Two cases (7.14%) were positive only by qPCR and only 1/28 (3.57%) case was positive by CISH. In the control group 9/33(27.27% were positive by both techniques, 23/33 (69.7%) were negative by both techniques and only 1/33 (3.03%) was positive by CISH and negative by real time PCR. The calculated kappa (K) value for carcinoma group was 0.783 and for the control group it was 0.926. The inter-rater reliability was substantial for the carcinomas group and outstanding for the control group.

Agreement and Inter-rater Reliability Between the Detection of JCV Agnoprotein by qPCR and Detection of JCV by CISH

Among carcinoma group, 11/28 (39.29%) cases were positive for JCV by both methods, and 12/28 (42.86%) cases were negative by both techniques. Only four out of 28 (14.28%) cases were positive by CISH and negative by PCR and 1/28 (3.57%) case was positive by PCR and negative by CISH. In control group 23/33 (69.7%) were negative by both techniques, 5/33 (15.15%) were positive by both of them, whereas 5/33 (15.15%) were positive by CISH technique only. The agreement between the 2 tests was moderate for both the carcinoma group and the control group. The calculated kappa (K) value for the carcinoma group was 0.646 and for the control group it was 0.582.

The mean age of patients with colorectal carcinoma was (52.85 ± 11.74) years and the frequency of cases were decreases with young age. Similar results were observed by (Zuhair R Al-Bahrani, and Al-Bahrani 2014), they cited that the average age for colorectal carcinoma was 56.83 year for males and 53.65 year for females. The highest frequency of cases was observed within age group of 50-59 years. This was a little bit different from another study in Iraq which found that the highest frequency was among 60-69 years of age (A.Majid, Shakir, and Mahmmod 2009; Iraqi-Cancer-Registry 2012).

The sex specific incidence in colorectal carcinoma showed a slight preponderance in males with ratio of male to female (1.8:1) and this was consistent with other Iraqi study Majid et al., (2009) who reported that male to female ratio was (1.6:1).

Non-mucinous adenocarcinoma was the most frequent histological type in this study which is compatible with other studies. Ahmed et al., (2010) and Hana et al., (2015) both published that 90% of cases were of non-mucinous adenocarcinoma. The most frequent histological grade was moderately differentiated adenocarcinoma (75%). Although in different frequencies, moderately differentiated adenocarcinoma was also the most common grade and represented (53%), (66.7) % and (90%) in different other studies (A.Majid et al. 2009; Abdulhussain, and Othman 2012; Hana, Azhar A.F. Al-Attraqchi, and Khattab 2015).

According to site of tumor (46.4%) were found in the LT. colon, (28.6%) were found in the rectum while (25%) of cases were from RT. colon. Hana et al., (2015) reported similar order but with different frequencies. They reported 56.7% in left colon, 33.3% in Rt. side and 10% in the rectum.

T-Ag gene a non-structural protein was selected because of its potential to bind and inactivate tumor suppressor protein p53 and pRb (Feild et al. 2001). The late accessory gene encoding agnoprotein of JCV was selected because it was suggested previously by Coello et al., (2010) to be contributing to colonic tumorigenesis for its potential to inhibit colonic epithelium differentiation just like inhibiting oligodendroglia differentiation. Although his observation awaited further work. Agnoprotein dysregulates the cell cycle and interferes with the pathways involving in DNA repair and chromosomal stability (Darbinyan et al. 2002; Darbinyan et al. 2007)

The results demonstrated the amplification of JCV T Ag gene region in 57% of carcinoma group and 27.3% of the control group. The gene encoding agnoprotein of JCV was also detected in 42.85% of colonic

carcinoma biopsies and 15.15% of the control group. The amplification results in the present study revealed higher copy number (viral load) of the JCV in colonic cancer tissue specimens than those of the control in both tests. In their work, other researchers also found higher viral load in carcinoma group than control (Hori et al. 2005; Mou et al. 2012).

In each of the amplification real time PCR tests conducted in the present work, there was a significant difference between the carcinoma group and the control group. However, the available data don't show the usefulness of any of the tests as sensitive test for screening for JCV in colonic specimens. On other hand, the amplification of agnoprotein gene region offered a better specificity than that of T Ag gene. Yet the viral load results in both the carcinoma group and control group were lower than those of the T Ag gene test.

The results also showed that there are 12 out of 16 colonic cancer cases positive for T Ag gene amplification were also positive for agnoprotein gene. This explains the substantial and acceptable agreement and inter-rater reliability between the 2 tests (Cohen, and Bartko 1994) which means that any of the available tests can be used to prove the presence of JCV in colorectal carcinoma as laboratory conformation.

Detection of JCV by Chromogenic In Situ Hybridization

Best to the present knowledge, this is the first study in Iraq which confirms the role of JCV in colorectal carcinoma using CISH. In virology this technique has earned its place in the experimental and clinical study of viral diseases it is considered the most sensitive and specific test for detection of certain viral agent in diseased tissue and tumors.

Table.1 Frequency distribution of T-Ag gene in study groups

		T Ag gene by PCR		Total
		Positive	Negative	
Study groups	Carcinoma	16 57.1%	12 42.9%	28 100.0%
	Control	9 27.3%	24 72.7%	33 100.0%
Total		25 41.0%	36 59.0%	61 100.0%

P=0.018

Table.2 Comparison of Viral Load Between Study Groups According to T Ag Gene Amplification by Real Time PCR

JCV T-Ag gene	Carcinoma group	Control group
Mean viral load	416.937	229.866
Standard Deviation	217.779	111.492
Standard Error of the Mean	56.230	39.418
Total Numbers :	16	9

P= 0.025

Table.3 Frequency Distribution of Agnoprotein Gene among Study Groups

Agnoprotein gene		Agnoprotein gene		Total
		Positive	Negative	
Study groups	Carcinoma	12 42.9%	16 57.1%	28 100.0%
	Control	5 15.2%	28 84.8%	33 100.0%
Total		17 27.9%	44 72.1%	61 100.0%

P= 0.016

Table.4 Comparison of Viral Load Between Study Groups According to JCV Agnoprotein Gene Amplification by Real Time PCR

JCV Agnoprotein gene	Carcinomas group	Control group
Mean viral load	317	152.94
Standard Deviation	129.121	105.149
Standard Error of the Mean	38.931	52.574
Total Numbers :	12	5

The P= 0.024

Table.5 Detection of JCV by CISH Technique in Study Groups

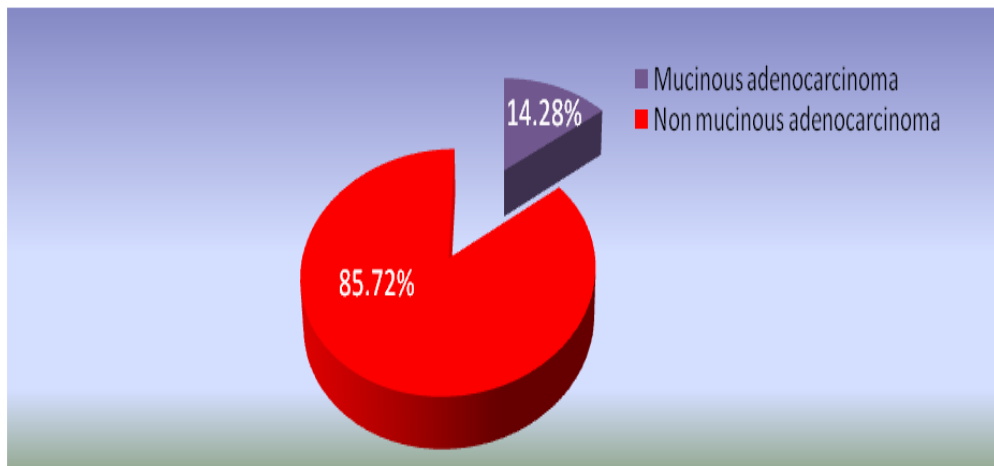
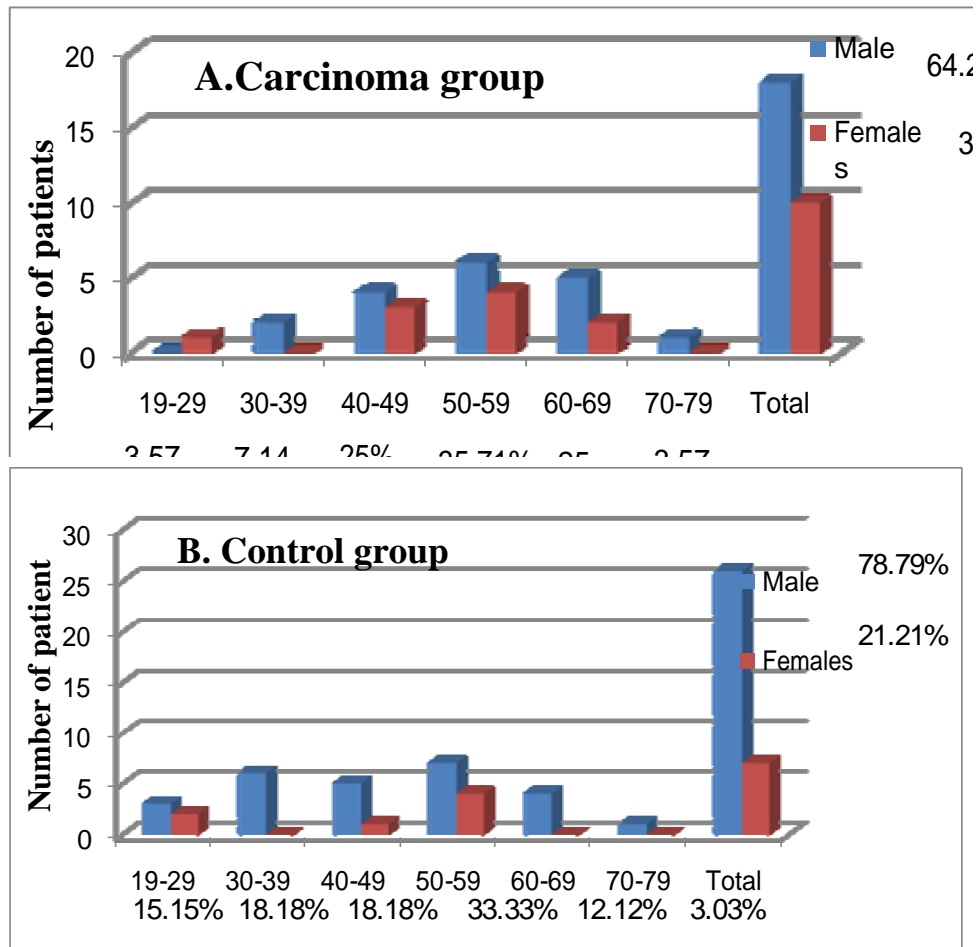
			JCV CISH		Total
			Positive	Negative	
Study groups	Carcinomas	% within JCV CISH	15	13	28
			53.6%	46.4%	100.0%
	Control	% within JCV CISH	10	23	33
			30.3%	69.7%	100.0%
Total			25	36	61
			41.9%	59.1%	100.0%

P= 0.065

Table.6 JCV DNA Expression by CISH Technique among the Study Groups

Variable	Normal No. (%) 33 (100%)	CRC No. (%) 28 (100)	p- Value
Intensity			
Low	7 (21.21%)	4 (14.29%)	P=0.020
High	3 (9.09 %)	19 (39.28%)	
Percentage score			
Score 1	8 (24.24%)	5 (17.86%)	P=0.014
Score 2	2 (6.06%)	10 (35.71%)	
Pattern of involvement			
Punctate	0 (0%)	9 (60%)	P=0.002
Diffused	10 (100%)	6 (40%)	
Signal location			
Glandular	6 (60%)	10 (66.7%)	NA
Stromal	0 (10%)	0 (0%)	
Glandular and stromal	3 (30%)	5 (33.3%)	

Figure.1 Age and sex Distribution of the Study Groups. A: Carcinoma, B: Control



Figure(2) Pie chart: Histological types of colorectal carcinoma in carcinoma group

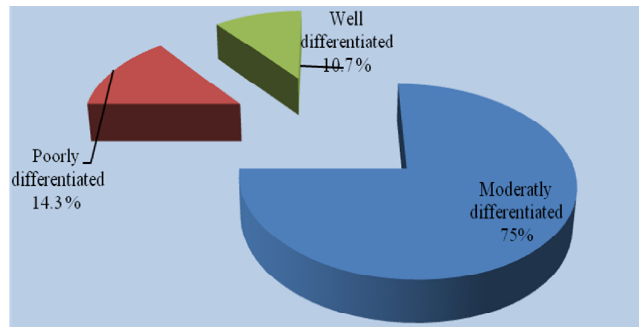


Figure 3: Pie chart show distribution of patients with adenocarcinoma according to tumor grade

Figure 4

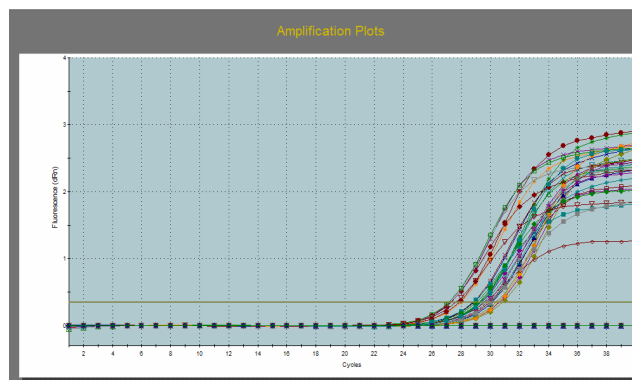
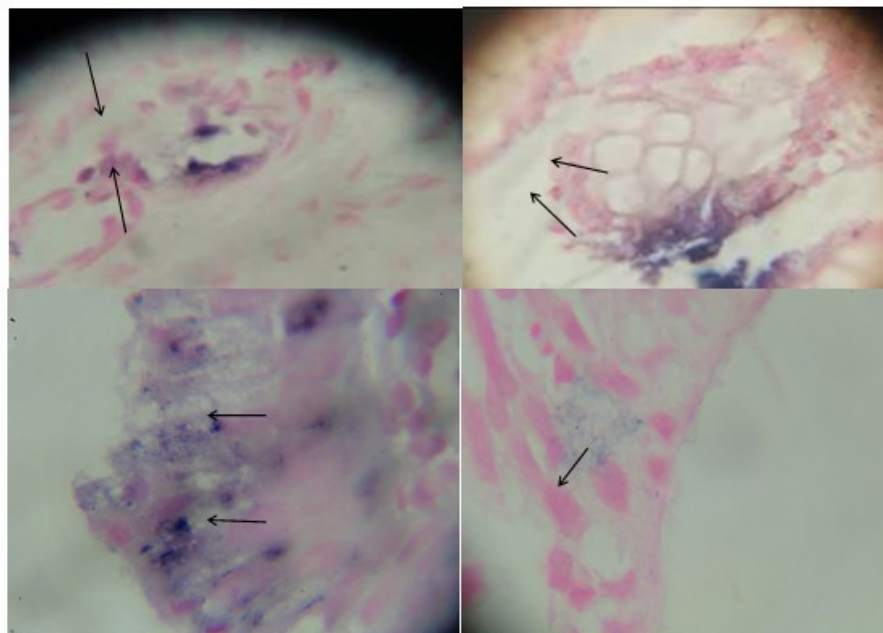


Figure.4-10 Different Colonic Biopsies Subjected to CISH Technique for the Detection of JCV



In the present study and by CISH technique 53.6% of the carcinoma group showed positive results to JCV DNA, while 31.3% of the control group showed positive results, no statistical significant difference was noticed. In Egypt, Samaka et al., (2012) found that 40.1% of carcinoma group were positive for JCV using CISH technique, 25% of the adenoma group and 20 % of normal control group. These mild differences could be due to different sample size, technical consideration, type and source of probe and kit used in the CISH technique as well as possible different prevalence. The probe which was used in the present study constitute of whole viral genome.

In the present study, both high intensity and high percentage scores were significantly linked to carcinoma group. Similarly the pattern of CISH signals was significantly linked to carcinoma group. These observations strengthen the role of the virus in the pathogenesis of carcinoma process. The punctate pattern is an indication of viral genome integration in the cellular DNA, which is an irreversible process and associated with the production of transforming protein rendering the product carcinogenesis inevitable (Zubillaga-Guerrero et al. 2013).

The distribution of CISH signal according to stromal and glandular involvement was determined in this study. The highest frequency of cases revealed glandular involvement among the study groups. Although, no statistical significant difference was observed, Smaka et al., (2012) reported 40% of CRC cases with glandular involvement, while 26% of cases revealed stromal involvement. They did not find a confined observation.

The presence of viral signal in glandular

localization indicates its presence in the epithelial cells. While this is associated with a stromal or blood vessels involvement a spread of the virus through blood stream a phenomena indicating its spread from another place possibly an extra colonic site. Recently, the earlier infection by the virus which can involve many sites in the body (Jiang et al. 2009).

In conclusion, the presence of JCV in tissue of colorectal carcinoma was more frequent than that in none carcinoma tissue. The viral load of JCV was higher in colorectal carcinoma compared to normal colonic tissue and with significant difference among study groups. Suggest that JCV may play a role as an etiology for colonic carcinogenesis.

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