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Role of the JC Polyomavirus (JCV) and BK Polyomavirus (BKV) in the colorectal cancer of some Egyptian patients

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Abstract

Although several cases and case-control studies reported the prevalence of JC polyomavirus (JCV) and BK polyomavirus (BKV) in tumor tissues of colorectal cancer (CRC) patients as well as in control samples; however, many recent studies did not show the prevalence of these viruses in the CRC patients. A total of 50 clinical biopsies samples were collected from patients with and without CRC in the General surgery department, Faculty of Medicine, Ain Shams University, Egypt, with the approval of the research ethics committee. Current results showed the absence of the genomic DNA of both JCV and BKV in the tumor tissue samples, adjacent-tumor normal mucosa samples from CRC patients, and also in the normal mucosa samples collected from the non-CRC patients. Moreover, results of the semi-nested PCR and real-time PCR are similar, and confirmed that JCV and BKV are not prevalent in the Egyptian CRC patients. There are no promising results for further investigation of the expression of the large T-Ag protein, and any other related proteins in the tumor cells. Based on the present results, no evidence supports the association between the JCV, BKV and CRC in the Egyptian patients. For the best of our knowledge, this is the first case-control study on the prevalence of JCV and BKV in the Egyptian CRC patients. The aim of the current study was to detect the presence of JCV and BKV genomic DNA in the tissue samples, to support further studies on the role of these viruses in the CRC etiology and development.

Keywords: Polyomaviruses, Colorectal cancer, Duplex semi-nested PCR, Egyptian patients

1. Introduction

Colorectal cancer (CRC) is one of the most important human cancers. In 2018, The International Agency for Research on Cancer (IARC) provided a new estimation of the incidence and mortality rates of cancer for both males and females worldwide. According to Bray *et al*., (2018), CRC was the third leading cause of cancer and the second leading cause of cancer deaths among all sexes and all ages,

accounting for approximately 1.8 million new cases and 881,000 deaths. In Egypt, different centers show a similar picture of the CRC, it has no age prediction and more than one-third of CRC tumors affect the young population. The high prevalence of CRC in the young people cannot be attributed to bilharziasis, and cannot be explained on a hereditary basis. Previous study of Abou-Zeid *et al*., (2002) reported that the predisposing adenomas were rare, and the disease was usually presented at an advanced stage.

Infectious agents mainly viruses are accepted as causes of cancer worldwide. The study of Pagano *et al*., (2004) revealed that these viruses account for most of the common malignancies (up to 20 %) of all cancers including; Human papillomavirus (HPV), Human polyomaviruses (HPyVs; JCV, BKV, SV40), Epstein-Barr virus (EBV), Kaposi's Sarcoma Herpes virus (KSHV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Human T-cell leukemia virus, (HTLV1), and Gastric carcinoma (Helicobacter pylori). Moreover, Lee and Langhoff, (2002); Damania, (2007) reported that unlike the other oncogenic viruses, the polyomaviruses oncogenesis were still a subject of controversy and were undergoing increasing researches.

Human polyomaviruses (HPyVs) are non-enveloped small virions of the family Polyomaviridae, with icosahedral symmetry and double-stranded circular DNA of molecular weight about 5 kb. According to Ahsan and Shah, (2002), the viral genome encodes early nonstructural transforming proteins (T antigens: T-Ag and t-Ag), and late structural capsid proteins (i.e. Vp1, VP2, VP3, and agnoprotein). JCV and BKV are the most prevalent members of the HPyVs, and have detectable levels of antibodies in most adult individuals. They were of high prevalence in water and wastewater worldwide, and were thought to be transmitted via a fecal-oral rout (Moens *et al*., 2013). BKV and JCV primary infections are asymptomatic or with only mild respiratory symptoms. Boldorini *et al*., (2011) showed that the infections occur early in childhood, and the viruses persist latently in various organs until they can reactivate when there is a deficiency in the immune system. These JCV and BKV were linked to several clinical manifestations such as the progressive multifocal leukoencephalopathy (PML) (Molloy and Calabrese, 2009), hemorrhagic cystitis (HC), bone marrow transplantation (BMT) recipients, and BKV-associated nephropathy (BKVN) in renal transplants (Nickeleit *et al*., 2003).

Recently, Prado *et al*., (2018) demonstrated that although some polyomaviruses have been linked to the development of various cancers; however, present evidences support the role of Merkel cell carcinoma polyomavirus (MCPyV) only as a human carcinogen. The MCPyV is considered as a group 2A carcinogen (probably carcinogenic to humans) and is known to cause Merkel cell carcinoma (MCC), while JCV and BKV are considered a group 2B carcinogen (possibly carcinogenic to human) (IARC. 2014). On the other hand, Bollag *et al*., (1989); Prado *et al*., (2018) attributed the oncogenic potential of the polyomaviruses to the ability of their transforming proteins (T antigens) mainly the large T antigen (T-Ag), to transform the human cells *in vitro* and *in vivo*. Gan and Khalili, (2004); Delbue *et al*., (2017) pointed that the T-Ag binds to and interacts with the tumor suppressor proteins p53 and pRb which regulates the cell cycle and proliferation, thus driving the infected cells to enter the S phase. In addition, this antigen interferes with the other cell signaling pathways such the Wnt signaling pathway. Several studies focused on the roles of JCV and BKV in the development of cancer through detection of the viral DNA or protein products in the tumor tissues of the cancer patients, in comparison with the control tissues. However, they may be either adjacent to the non-tumorous healthy tissues of the same cancer patients, or to tissues from the same organ site of the non-cancer patients (IARC. 2014). In Egypt, there is no data on the prevalence of JCV and BKV in the CRC patients, and on their possible roles in the etiology and development of the tumor. This is the first study to investigate the presence of the genomic DNA of JCV and BKV in the tumor and normal mucosa of the Egyptian CRC

patients, in an attempt to identify the roles of these viruses in the etiology of CRC.

2. Material and methods

2.1. Samples collection

With approval of the research ethics committee, fifty clinical samples were collected from patients with and without colorectal cancer (CRC) in General surgery department, Faculty of Medicine, Ain Shams University, Cairo, Egypt, during the period from December 2015 to March 2017. About 48 samples were collected from patients with CRC including; 24 biopsies from tumor tissues, 24 biopsies from tumor-adjacent normal mucosa of the same patients at least 10 cm far from the gross of cancer, confirming that all the cells were intact and free from any contaminating tumor cells. In addition, 2 biopsies samples were collected from the normal mucosa of patients without CRC. All these samples were of weight about 100 mg, collected in sterile containers and then stored in -80°C till further use.

2.2. Extraction of the viral DNA

Extraction of the viral DNA was carried out using QIAamp DNA Mini Kit (QIAgen, Inc.) following instructions of the manufacturer. The total nucleic acids were eluted in 200 µl of elution buffer and then stored in -80°C. Standard precautions were followed to avoid contamination.

2.3. Detection of the JCV and BKV using Duplex semi-nested PCR

Amplification of the JCV and BKV genomes was performed using semi-nested PCR (snPCR) described by Biel *et al*., (2000), and modified to perform duplex snPCR to detect the JCV and BKV genomes in the same reaction. The duplex PCR was designed to target a conserved genomic region of the large T-antigen protein (T-Ag) of both JCV and BKV with amplicon sizes of 199 bp and 246 bp, respectively. Approximately 10 µl aliquot of the extracted nucleic acids was used for each test. Standard precautions were applied to reduce the probability of samples cross contamination.

First round of PCR amplification was carried out using primers PV-SNFOR and PV-BACK as forward and reverse primers, respectively. The reaction mixture (50 µl) contained 25 µl of COSMO PCR RED Master Mix (Willowfort, Birmingham, UK), 2.5 µl of forward and reverse primers (10 µM), 15 µl of Nuclease-free water (Promega-USA), and 5 µl of DNA template. The amplification was performed in a programmable Thermal cycler (Techne Genius FGEN02TP) using the following conditions: initial denaturation at 94°C for 3 min., 30 cycles of denaturation at 94°C for 20 s, annealing at 53°C for 20 s, extension at 72°C for 30 s; and a final extension at 72°C for 5 min. About 3 µl of the first PCR product was used as a template for the second amplification round, which was carried out as a duplex PCR using PV-BACK as the reverse primer; JC-FOR and BK-FOR as the forward primers for JCV and BKV, respectively. The reaction was carried out under the same conditions except for raising the annealing temperature to 58°C. Appropriate negative and positive controls were included in each test run. The oligonucleotide primers (Willowfort, UK) were designed based on the nucleotide sequence described by Biel *et al*., (2000). The Primers nucleotide sequences used in this study are described in Table 1.

The results were analyzed using Agarose gel electrophoresis, visualized using Ultraviolet (UV) transilluminator at wavelength of 312 nm, and photographed by the gel documentation system (InGenius3, Syngene). The amplified DNA fragments were identified by comparison with the molecular size marker DNA (100 bp) Ladder (Cleaver Scientific Ltd, Rugby, Warwickshire, UK).

2.4. Quantitative Real-time PCR

To improve the sensitivity of detection of the JCV and BKV and confirm results of the duplex snPCR, quantitative real-time PCR (qPCR) was carried out for

Table 1. The oligonucleotide primers used for PCR amplification and sequencing of the JCV and BKV

Virus	Primer	Amplification reaction[a]	Region[b]	Location (nt)[c]		Sequences
				BK[d]	JC[e]	
JCV&BKV	PV-BACK	Q First-reverse Sn-reverse	T-Ag	4548-4567	4408-4427	5′-GGTGCCAACCTATGGAACAG -3′
JCV&BKV	PV-SNFOR	First-forward	T-Ag	4307-4329	4170-4192	5′-TCTTTAGT**R**GTATACACAGCAAA -3′
JCV	JC-FOR	Sn-forward	T-Ag		4229-4248	5′-GAGGAATGCATGCAGATCTA -3′
BKV	BK-FOR	Sn-forward	T-Ag	4322-4338		5′-ACAGCAAAGCAGGCAAG -3′
JCV&BKV	PV-QFOR	Q	T-Ag	4342-4365	4205-4228	5′-TCTATTACTAAACACAGCTTACT -3′

Where; [a]Sn: semi-nested PCR; Q: quantitative real-time PCR

[b]T-Ag: large T antigen; [c]nt: nucleotide

[d]Nucleotide positions are based on the sequence of the HPyV BK genome (Accession no. J02038)

[e]Nucleotide positions are based on the sequence of the HPyV JC genome (Accession no. J02226)

R: A/G (Biel *et al*., 2000)

all the samples as described by Biel *et al*., (2000), using PV-QFOR and PV-BACK primers (Table 1). The PCR amplification was carried out in 20-µl reaction mixture containing 10 µl of Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific™, Waltham, Massachusetts, U.S.), 0.5 µl of forward and reverse primers (10 µM), 7 µl of Nuclease-free water (Promega-USA), and 2 µl DNA template. Amplification was performed using a real-time system (Rotor-Gene Q, QIAgen) with the following conditions: initial denaturation at 95°C for 10 min., 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s.

A positive sample of HPyV from unpublished work was used to carry out the PCR assay using PV-QFOR and PV-BACK primers. The PCR product was then purified using DNA purification kit (DNA Clean and Concentrator™-25, Zymo Research, California, USA). The nucleic acid concentration was measured by nanodrop and used to prepare 10-fold serial dilutions (101-108 copies/ ml), to construct a standard curve for possible quantification of the positive samples. All the clinical samples were tested twice using the snPCR and the real-time PCR to obtain more doubtless results.

3. Results

3.1. Prevalence of the JCV and BKV in the clinical samples

Results of amplification of both JCV and BKV genomes using the Duplex snPCR and analysis of these results using the agarose gel electrophoresis, demonstrated that 100 % (50/50) of the samples are negative for both of the JCV and BKV genomes. The viral genomes of both viruses are not detected neither in the tumor tissues nor in the normal mucosa of all the tested CRC patients. All samples were confirmed to be free from DNA of both viruses by comparison with positive and negative controls included in all amplification reactions. The target T-Ag sequence amplicons of 199 bp and 246 bp of JCV and BKV; respectively, are not detected in any of the tested samples (Fig. 1). Both viruses are not prevalent in colorectal tissues among both genders and all ages included in the present study.

Fig. 1. Agarose gel electrophoresis of DNA of the 9 tested samples is presented in lanes 3-11. Where; L: DNA ladder; Lane 1: positive controls (JCV, 199 bp) and (BKV, 246 bp); Lane 2: negative control

3.2. Quantitative real-time PCR

Although qPCR technique is more sensitive than snPCR in detection of DNA; however, results of the qPCR confirmed those of the snPCR that none of the examined samples of the CRC patients recorded the detection of the DNA genomes of the JCV or BKV, in comparison with the positive controls included in all amplification reactions.

4. Discussion

Many authors studied the association between the human polyomaviruses (JCV and BKV) and the CRC, in attempts to clear the controversy about the role of these viruses in the etiology of cancer worldwide (Campello *et al*., 2010; Coelho *et al*., 2013; Ksiaa *et al*., 2015; Sarvari *et al*., 2018). These studies were either case studies that included tumor tissue samples from CRC patients only, or case-control studies including tumor tissue samples from CRC patients. While the control samples might be tumor-adjacent normal mucosa samples from CRC patients, or normal mucosa samples from non-CRC patients; however, the

patients undergo colonoscopy for other gastrointestinal disorders. The hypothesis of these studies was that if JCV and BKV play roles in causing these cancers, the virus DNA or the expressed proteins will be detected more frequently and in high numbers in the tumor tissues than in the control tissues. However, IARC. (2014) reported that presence of the virus in the tumor tissues in higher numbers than in the control tissues does not support its causative role in the etiology and development of cancer.

The Duplex PCR used in the current study gives an advantage for detection of the JCV and BKV in one reaction. This technique is designed to target a conserved genomic region of the large T antigen protein (T-Ag) of both of JCV and BKV, and it gives different amplicon sizes of 199 bp and 246 bp, respectively (Biel *et al*., 2000).

Several studies of Newcomb *et al*., (2004); Giuliani *et al*., (2008); Campello *et al*., (2010) reported that about 233, 66, 94 of CRC patients in USA, Italy, and North-East Italy; respectively, recorded 0 %, 0.4 %, and 0 % prevalence of JCV in tumor tissues, in tumor-adjacent normal mucosa, and in normal mucosa, respectively. A recent study of Sarvari *et al*., (2018) in Iran pointed the detection of DNA of the JCV in only one out of seventy adenocarcinoma colorectal tissues, while did not detect it in all adenomatous and normal colorectal tissues. On the other hand, the DNA of BKV has been detected in 9 % (6/66) of the tumor tissue samples examined in in Italy (Giuliani *et al*., 2008). In the North-East of Italy, Campello *et al*., (2010) did not detect the same viral genome in the tumor tissues of about 94 CRC patients and in 91 of non-CRC patients.

In contrast to the present results, six case-control studies in USA, Taiwan, China, Portugal, and Tunisia (2 studies) reported the high prevalence of JCV of 77 %, 84.4 %, 40.9 %, 90 %, 58.1 % and 40 % in the tumor tissues, and of 72 %, 0 %, 24.8 %, 48 %, 14.6 %, 0 % in tumor tissues adjacent to the normal mucosa, respectively (Goel *et al*., 2006; Lin *et al*., 2008; Mou *et al*., 2012; Coelho *et al*., 2013; Ksiaa *et al*., 2015; Toumi *et al*., 2017). However, The DNA of BKV has not been detected in any of the examined tumor and tumor-adjacent tissues (Goel *et al*., 2006).

In previous studies, Goel *et al*. (2006); Lin *et al*., (2008) investigated the nuclear expression of the large T antigen (T-Ag) protein, and recorded its expression in 43 % and 63.6 % of the tumor tissues, respectively. In the first study, the T-Ag protein was not expressed in all the tumor tissues, and only 43 out of 77 of the JCV DNA positive tissues contained this T-Ag protein. However, the T-Ag has not been expressed in any of the adjacent tissue samples in both studies. The study of Ksiaa *et al*., (2015) recorded a significant correlation between the nuclear expression and accumulation of β-catenin and p53 in the tumor cells, in addition to the presence of JCV in these tumor cells. Accumulation of the β-catenin in the nucleus stimulated by the viral T-Ag, activates transcription of a series of cellular genes that are involved in rapid cell proliferation.

Despite of all these studies and due to the variation in results, no clear association has been recorded between JCV, BKV and the CRC patients. Thus the controversy about their roles in the etiology and development of cancer still exists, and needs deep investigation.

Conclusion

JCV and BKV have no prevalence in the Egyptian colorectal cancer patients, and thus there is no evidence about their roles in the etiology and development of CRC. The observed variation in the results across the different studies may be attributed to several reasons including; the differences in sensitivity of assays used in each study, possible contamination during extraction and amplification of the DNA, differences in the prevalence of JCV and BKV, and risk factors involved in viruses-associated carcinogenesis among the different populations and geographical areas. Based on the different and non-

conclusive results, the association between these viruses and the CRC remains controversial and needs more deep investigation.

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Conflict of interest

The authors declare no conflict of interests.

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Ethical approval

The protocols used in the study were approved by the Ethics Committee of the Faculty of Medicine, Ain Shams University. The patient's consents and statement of protection of the patient's privacy are provided.

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