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Molecular Detection of SV40, BKV and JCV in Esophageal and Colorectal Cancer Patients in Khartoum State, Sudan

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Methods: Paraffin embedded blocks of tumor specimen from 56 colorectal cancer patients and 25 from esophageal cancer patients were collected from Khartoum teaching hospital and Medical Military Hospital, Sudan. Multiplex nested PCR was used to investigate the presence of BKV, JCV, and SV40 viruses in these specimens.

Results: BKV, JCV, and SV40 DNAs were detected in 11 out of 56 (19.64%) colorectal cancer samples and 3 out of 25 (12%) esophageal cancer samples, . Out of 56 patients diagnosed with colorectal cancer patients, 17%, 1.75% were found positive for SV40, JCV respectively, but no BKV positive samples were detected. Out of 25 patients diagnosed with esophageal cancer patients, 8%, and 4% were found positive for JCV, and BKV respectively, but SV40 was not detected in any of these samples.

Conclusion: The incidence of BKV, JCV, and SV40 in colorectal and esophageal cancer patients in Khartoum State, was documented through the molecular detection of BKV, JCV, and SV40 indicating high prevalence rates for SV40 among colorectal cancer patients in Khartoum State. Detection of BKV, JCV, and SV40 using multiplex nested PCR was established. Generally, these findings are useful for future studies since there is little information available about BKV, JCV, and SV40 infection in Sudan.

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I. Introduction

Polyomaviruses are a family of non-enveloped DNA viruses with icosahedral capsids containing small, circular, double-stranded DNA genomes. Polyomaviruses have been isolated from multiple animal species including humans, monkeys, rodents, and birds. Each polyomavirus exhibits a very limited host range and does not usually productively infect other species (Fields et al., 1996, Imperiale, 2001).

The polyomavirus family includes several human viruses, JCvirus (JCV) and BK virus (BKV), both of which were isolated in 1971 from immune compromised patients (Shah, 1996). JCV was recovered from the brain of a patient with the initials J.C. who died of progressive multifocal leukoencephalopathy (PML), a demyelinating disorder of the central nervous system (CNS) (Padgett et al., 1971). BKV was isolated from the urine of a Sudanese renal transplant patient (with the initials B. K.) who developed ureteral stenosis and was shedding inclusion-bearing epithelial cells in his urine (Gardner et al., 1971).

In the late 1950s and early 1960s, millions of people around the world were inadvertently exposed to a third polyomavirus, Simian virus 40 (SV40) of rhesus macaques (*Macaca mulatto*), due to administration of contaminated polio vaccines (Mortimer et al., 1981). This virus, Simian virus 40 (SV40), is a natural infectious agent in rhesus macaque. Recent studies revealed the presence of SV40 DNA in healthy individuals that were never vaccinated with contaminated vaccines or those who had never been in contact with monkeys. Seroepidemiological studies revealed that up to 15% of the human population contains antibodies against Simian virus 40, thus supporting the possibility that SV40 can spread in human by means of horizontal infection and vertical transmission (Martini et al., 2007).

The most studied human polyomaviruses are BK virus and JC virus. The route of infection remains unknown, but respiratory, oral, body fluids, and renal tansplacental transmission has been suggested (Knowles, 2006). BKV is a nephrotropic virus, but nucleic

acid sequences and proteins can be detected in other tissues like blood, brain, liver, heart, lung and gonads (Rekvig and Moens, 2002), while JCV nucleic acid can be found in the kidney, blood, urogenital system cells and the gastrointestinal tract (Dörries, 1984).

Adult seroprevalence for BKV and JCV is very high: more than 90% of the adult population is seropositive for BKV (Knowles et al., 2003), while 50 to 80% of adults have antibodies to JCV (Knowles, 2006, Khalili et al., 2007).

Interestingly, the antibody titer against BKV decreases as the age increases, while that of JCV remains relatively unchanged (Knowles, 2006, Dörries, 1984).

The primary infection with BKV and JCV seems to be asymptomatic and the virus establishes a harmless life-long latent infection in the host, but reactivation of the virus in immunosuppresed individuals can lead to illness. BKV is associated with nephropathy (PyVAN) in renal transplant patients and hemorrhagic cystitis (PyVHC) in bone narrow transplants (Fleischmann, 2009, Hirsch and Snydman, 2005).

JCV is causative agent of PML. a fatal progressive demyelinating disease of the central nervous system due to viral replication in the oligodendrocytes (Rekvig et al., 1997).

The polyomaviruses JCV, BKV, and SV40 have been implicated in several human diseases and are undergoing increased scrutiny as possible cofactors in human cancer (Ahsan and Shah, 2002). These viruses can induce tumors in several rodent species, and can be detected with higher frequency in certain tumors compared to the corresponding healthy tissue (Moens et al., 2011).

The first study of polyomavirus in Sudan was done in 2016, in symptomatic kidney transplant recipients (Helibi et al., 2016). The most recent study was done in 2017, in patients with Non-Hodgkin's Lymphoma (NHL) (Isam, 2017).

Antibody assays are commonly used to detect presence of antibodies against individual viruses. (Drachenberg et al., 2005), but is rarely used to detect primary infection since most primary infections occur asymptomatically in early childhood (Flaegstad. and Traavik, 1985, Bogdanovic et al., 1994), detection of polyomaviruses by PCR or multiplex nested PCR is more sensitive and useful, although it is possible to use electron microscopy and virus isolation (Padgett et al., 1971, Schmitt et al., 2011).

This present work aimed to provide a better understanding for the role of BKV, JCV and SV40 in colorectal and esophageal cancer patients in Sudanese populations and update the information regarding the disease situation due to lack of diagnostic tools in the Sudan.

II. Materials and Methods

a) Study area

This study was conducted in Khartoum Hospitals (Royal care Hospital and Medical Military Hospital) during the period from, October 2018 to July, 2019.

b) Study design

This study is descriptive, cross- sectional study.

c) Ethical review

The study was approved by the Ethical Review Committee (ERC) of Al Neelain University, Faculty of Medical Laboratory Sciences. Informed consents were obtained from the patients.

d) Data collection method and tools

Through a structured questionnaire, information on age, gender, and type of tumor and place of sample collection was recorded.

e) Patient's inclusion criteria and sample size

Paraffin embedded blocks tumor specimens from 81 Sudanese patients (65 colorectal cancer and 25 Esophageal samples taken from normal and pathological lesion) were collected in sterile Eppendorf tube and stored at room temperature until used for DNA extraction. most frequent lesions were adenocarcinoma.

f) Sample deparaffinization

Tissue samples were deparaffinized using xylene dissolution, in brief two of 20 μm sections were cut from each tissue sample block by the same person. To avoid cross-contamination, the microtome block was cleaned and the blade replaced between samples. All samples were deparaffinized by adding xylene for one hour and then washed by ethanol 100%, 80%, 60% and 40% conseeuctively then deionized water for 10 seconds for rehydration.

g) DNA extraction

DNA was extracted from rehydrated tissue by using DNA extraction Kit according to the protocol of the manufacturer (Analytikajena), Briefly, 20-μ sections of rehydrated sample was added to 560 μl buffer AVL, then incubated at room temperature for 10 minutes. Subsequently, 560 μl of ethanol (96- 100%) was added to the sample after which 630 μl of the resulting solution was applied to a column. A volume of 500 μl of AW1 and AW2 was added for washing and the nucleic acids were eluted with 60 μl AVE buffer and stored at -20°C until used.

h) Multiplex nested Polymerase Chain Reaction (PCR)

The multiplex nested PCR was done as described by Bergallo (Bergallo et al., 2007), The test was carried out with first-round PCR amplification using the outer primer pairs that are specific for large T

antigen gene to amplify a conserved DNA region of the large T antigen gene of JCV, BKV, and SV40.

The primers used consisted of forward primer 5′-TCYTCTGGNNTAAARTCATGCTCC-3′ and reverse primer, 3′- CAAGGTATCCAACCKTRGATWAA -5′. The reaction was performed in 25µl volume using Maxime PCR PreMix Kit master mix (Intron. South Korea). The volume included: 5µl master mix, 1 µl forward primer, 1 µl reverse primer, 5 µl extracted DNA and 13µl distilled water. The DNA was amplified in thermo- cycling conditions using PCR machine (Techno ,Japan) as follow: initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min and extension at 72°C for 1 min, with a final extension 72°C for 5 min.

The second round was carried out using a set of primers designed to obtain products of different size for each related virus, inner primer pairs used consisted of BKV Sense 5′- GAATGCTTTCTTCTATAGTATGGTATG -3′ and JCV Sense 5′- ATATTATGACCMCCAAAACCATG -3′ and SV40 Sense 5′- ATAATTTTTTTGTATAGTATAGTAGTGCA -3′ with reverse Polyomavirus Antisense 3′- CCTTTCAGRAAYCCCATAAGATGG-5′ The reaction was performed in 25µl volume using Maxime PCR PreMix Kit master mix (Intron. South Korea).

The volume included: 5µl master mix, 2 µl of primers mix included the inner primer pairs that mentioned above, 15µl distilled water and 3 µl of first-round PCR product, second round was performed under the following conditions: 94°C for 30 s, 56°C for 1min, 72°C for 30 s for 30 cycles with a final extension 72°C for 5 min.

i) Visualization of products

10 µl of the amplified product was subjected to direct analysis by gel electrophoresis in 2% Agarose, the gel was prepared by adding 1.6 g of Agarose to 75 ml 50X Tris Acetate EDTA buffer. The product was visualized by staining with 0.2 µg/ml Ethidium bromide using UV gel documentation system Biometra (Germany). The expected size of SV40, BKV and JCV amplicons were 135 bp, 353 and 189 respectively.

j) Data analysis

Collected data were analyzed using the statistical package of social science (SPSS, version 12.0) program. Chi-square statistical analyses was used to determine P value significance range.

III. Results

a) Detection of JCV, BKV and SV40 in colorectal and esophageal cancer patients

Eleven out of 56 (19.64%) colorectal cancer samples were analyzed, 10 of which (17.85%) were found positive for SV40, and 1(1.78%) was found positive for JCV, BKV was not detected in any of the samples. (Table 3.1).

Three out of 25 (12%) esophageal cancer samples were analyzed, of which 1/25 (4%) was found positive for BKV and 2/25 (8%) were found positive for JCV, one of these sample showed mixed infections with BKV and JCV, however SV40 was not detected in any of the esophageal cancer patients. None of 25 normal lesion samples were positive for JCV, BKV and SV40. (Table 3.1).

b) The association between gender and the presence JCV, BKV and SV40 in colorectal and esophageal cancer patients

According to the gender for colorectal cancer patients JCV was detected in 1/34 (2.9%) male and SV40 was detected in 6/34 (17%) males and 4/22 (18.2%) of females.

In esophageal cancer patients BKV was detected in 1/16 (6.3%) female and JCV was detected in 2/16 (12.5%) females.

There was no significant association between the gender and virus detection neither for esophageal cancer (P value = 0.428) nor for colorectal cancer patients (P value = 0.135). (Table 3.2, Table 3.3)

c) The association between the age groups and presence of JCV, BKV and SV40 in colorectal and esophageal cancer patients

Based on age group, the distribution of JCV in colorectal cancer patients were 1/33 (3%) in the age groups 31-60 years old, while SV40 distribution of positive samples showed 2/7 (28.5%) in age groups 18-30 years old; 5/33 (15.5%) in age groups 31-60 years old; 3/16 (18.75 %) in age groups 61-77 years old.

In esophageal cancer patients the age group distribution for JCV was 2/12 (16.6%) in age groups 31-60 years old, while distribution of BKV positive samples was 1/12 (8.3%) in age group 31-60 years old. No significant differences according to age were found (P value = 0.632) and (P value = 0.135) for colorectal cancer and esophageal cancer respectively. (Table 3.4), (Table 3.5).

Table 3.1: Detection of BKV, JCV and SV40 in colorectal and esophageal cancer

Polyomavirus	Colorectal	Esophageal		Total
		Normal	pathological	
BKV	0/56 (0%)	0/25 (0%)	01/25 (4%)	1/ 81(1.23%)
JCV	01/56 (1.75%)	0/25 (0%)	02/25 (8%)	3/81 (3.7%)
SV40	10/56 (17%)	0/25 (0%)	0/25 (0%)	14/81 (17.2%)

Table 3.2: Patients with colorectal cancer, classified as to gender and the type of Polyomavirus in Khartoum State, Sudan 2018 (n=56)

Polyomaviruses	Male	Female	Total
BKV	0/34 (0%)	0/22 (0%)	0/56 (0%)
JCV	1/34 (2.9%)	0/22 (0%)	1/56 (1.78%)
SV40	6/34 (17%)	4/22 (18.2%)	10/56 (17.85%)
Total	34/56 (60.7%)	22/56 (39.3%)	11/56 (19.6%)

(P value = 0.428)

Table 3.3: Patients with esophageal cancer, classified as to gender and the type of Polyomavirus in Khartoum State, Sudan 2018 (n=25)

Polyomaviruses	Male	Female	Total
BKV	0 /9 (0%)	1/16 (6.3%)	1/25 (4%)
JCV	0/9 (0%)	2/16 (12.5%)	2/25 (8%)
SV40	0/9 (0%)	0 /16 (0%)	0/25 (0%)
Total	9/25 (36%)	16/25 (64%)	3/25 (12%)

(P value = 0.135)

Table 3.4: Patients grouped by age and the detected Polyomavirus in colorectal cancer patients in Khartoum State, Sudan 2018

Age groups	Colorectal cancer patients			
	BKV	JCV	SV40	Total
18 – 30	0/7 (0%)	0/7 (0%)	2/7 (28.5%)	2/7 (28.5%)
31-60	0/33 (0%)	1/33 (3%)	5/33 (15.15%)	6/33 (18.1%)
61-77	0/16 (0%)	0/16 (0%)	3/16 (18.75%)	3/16 (18.75%)
Total	0/56 (0%)	1/56 (1.78%)	10/56 (17.85%)	11/56 (19.6%)

(P value = 0.632)

Table 3.5: Patients grouped by age and the detected Polyomavirus in esophageal cancer patients in Khartoum State, Sudan 2018

Age groups	Esophageal cancer patients			
	BKV	JCV	SV40	Total
18 – 30	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
31-60	1/12 (8.3%)	2/12 (16.6%)	0/12 (0%)	3/12 (25%)
61-77	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)
Total	1/25 (4%)	2/25 (8%)	0/25 (0%)	3/25 (12%)

(P value = 0.135)

IV. Discussion

Oncogenic viruses may contribute to human carcinogenesis favoring genetic instability and inducing chromosomal aberrations (Duensing and Münger, 2003), it is well established that BKV, JCV, and SV40 can cause cancer in laboratory animals (Walboomers et al., 1999), and all three polyomaviruses are associated with human tumors. (Ahsan and Shah, 2006) however the role of polyomaviruses BKV, JCV and SV40 is still controversial. (White and Khalili, 2004).

The present study focused on the molecular diagnosis of three human polyomaviruses (BKV, JCV, and SV40) in colorectal and esophageal cancer patients in Khartoum State, Sudan since little is known about the epidemiology of this three human polyomaviruses in Sudan in particular and in Africa in general.

The human polyomaviruses can persist in the host in a latent form and reactivate in the presence of immunosuppressive conditions. They are commonly associated with rejection of transplanted kidney (BKV) and progressive multifocal leukoencephalopathy (JCV). More recently, they have been linked to colorectal carcinogenesis. (Hori et al., 2005, Enam et al., 2002, Casini et al., 2005).

SV40 is a monkey virus that was probably introduced in the human population in the early 1960's by contaminated polio vaccines produced in monkey kidney cells where the virus can be present in a latent form. It probably continued to spread among humans through the sexual, haematogenic and orofecal routes, since it was found in urine and sewage samples. (Theodoropoulos et al., 2005, Li et al., 2002).

Colorectal cancer is one of the most common malignancies in developed countries. (Vastag, 2002), and this is the first report describing the presence of SV40 DNA in colorectal cancer in Sudan, we found that 17% of the sample were positive for SV40.These findings are similar of that reported by laura giuliani (2008) in Italy who reported that 15.1% of the colorectal cancer patients had the virus.

JCV was found positive in 1.75% of our clorectal cancer patients which is agreement with results 4.2% were positive for JCV DNA reported by El Hussein et al (2019) as well to that of Sarvari *et al* (2018) who reported low prevalence of 1.42% JCV DNA in Shiraz city, Iran, and lower percentage compared to that reported by Enam et al (2002) who reported extremely high rate of 81% in colon cancers and to that of Rencic et al.(1996) who reported detection rate of 81.2% in colonic biopsy samples. The lack of detection of JCV T-Ag however cannot rule out a "hit and run" mechanism as demonstrated by Ricciardiello *et al* (2003). in an *in vitro* model of colonic cells BKV, on the other hand, was not detected in colorectal cancer.

For the esophageal cancer we found JCV DNA in 2/25 (8%) samples which slightly differs from that reported (53%) by Del Valle et al., (2005), and Ahsan and Shah, 2006).In our positive samples single infection was present in 1 case and dual infections in the remaining case which also had BKV, normal lesion samples were negative for JCV.BKV and SV40. The high prevalence of infection and detection of BKV and JCV in tonsils suggested that the virus is transmitted mainly by the respiratory route. However, it has been reported that JCV can infect cells in the tonsils and can spread from there by replication in lymphoid cells. (Ahsan and Shah, 2006).

BKV and JCV DNA sequences and virions are also detected in raw urban sewage, (Del Valle et al., 2005, Bofill-Mas et al., 2001) suggesting also a fecal-oral route of transmission for these viruses. In the present study, SV40 was not detected in any of the esophageal cancer specimens, other study in Sudan showed that prevalence of BKV and SV40 in NHL patient which both males are more susceptible (Isam et al., 2017).

The study showed that there is no significant association with gender or age with the presence of these three human polyomaviruses in both in colorectal and esophageal cancer.

Finally; our study showed the feasibility of Multiplex nested PCR assay for detection and differentiation between JCV, BKV, and SV40 in cancer tissues and could be used for diagnostic purposes and epidemiological studies in the Sudan.

To our knowledge, this is the first attempt to detect JCV, BKV, and SV40 in colorectal and esophageal cancer patients in Sudan by using Multiplex nested PCR assay. The results obtained should call for wider surveillance at the national level in order to fully elucidate the true status and epidemiology of JCV, BKV, and SV40 in the country.

V. Conclusion

Incidence and existence of JCV, BKV, and SV40 in Sudan was documented through detection of these viruses DNAs in the tissue samples among colorectal cancer and esophageal cancer patients in Sudan, using Multiplex Nested PCR. Generally, these findings are useful for future studies since there is little available information about human polyomaviruses infection in Sudan.

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