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ORIGINAL ARTICLE

Frequency of BK virus genotypes in patients with colorectal cancer

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ABSTRACT

Background and Objectives: BK polyomavirus infection is prevalent and primarily asymptomatic, except for complications in kidney transplant recipients. Furthermore, its involvement in a tumorigenic family necessitates consideration in various malignancies such as urogenital tumors, prostate cancer, colorectal cancer (CRC), and brain cancer.
Materials and Methods: This investigation encompassed 50 specimens of colorectal adenocarcinoma tumors, 50 adjacent tissues, and 40 urine samples, with patients having a mean age of 61 years ± 12.4 years. The detection of BK virus DNA VP1 gene and genotyping were carried out through nested-PCR and sequencing techniques.
Results: Through the utilization of nested-PCR, BK virus DNA was identified in 15/50 (30%) colorectal tumor samples and 3/50 (6%) adjacent tissues (p-value = 0.008). Additionally, 6/40 (15%) urine samples exhibited positive results for BK virus DNA. Notably, among these findings, 9/15 BK virus positive tumor tissues (60%) and 3/6 BK virus positive urine samples (50%) were confirmed to be positive for BK virus subtype 4 (p-value < 0.001), whereas 2 tumor samples and 3 urine samples were attributed to BK virus type 1b2.
Conclusion: It is imperative to enhance one's understanding of the etiological and risk factors pertaining to cancers. The present findings offer substantiation of a potential correlation between BK virus infection and colorectal cancer. BK virus genotype 4 was found to be dominant among the CRC patients in this study.

Keywords: BK polyomavirus; Colorectal cancer; Urine; Genotypes

INTRODUCTION

According to the GLOBOCAN database, colorectal cancer (CRC) ranks as the third most frequently diagnosed malignancy (10.0%) and constitutes the second leading cause of cancer-related mortality (9.4%) globally, following lung cancer (1). In Iran, the incidence of CRC was projected to be 9.1% in year 2020 (2).

A multitude of factors, including alterations in dietary habits, an increasingly sedentary lifestyle, an ascending Human Development Index (HDI), advancing age, inflammatory bowel disease (IBD), tobacco use, alcohol consumption, elevated Body Mass Index (BMI), familial cancer history, and diabetes, contribute to the etiology of colorectal cancer

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(1, 3, 4). The potential direct or indirect influence of microbial infections on the pathogenesis of CRC is receiving increasing attention from researchers (5). BK Polyomavirus (BKPyV) Bk virus has also emerged as a potential contributor to tumorigenesis (6-8) or as a co-factor for established oncoviruses such as human papillomaviruses in various cancers (9-11).

The BK virus was initially identified in the urine of a male patient with chronic renal conditions by Gardner et al. (12). The BK virus is classified as a non-enveloped double-stranded DNA virus that is a member of the Polyomaviridae family. Strains of BK virus have been categorized into four distinct genotypes, specifically 1 through 4. Genotype 1 is regarded as the most prevalent globally, accounting for approximately 80%, with genotype 4 following at 15% (13). The BK virus, recognized as a ubiquitous pathogen, is predominantly transmitted during childhood or early adulthood via the fecal-oral route. Additional routes of BK virus transmission include blood transfusions, respiratory secretions, seminal fluid, organ transplants, and transplacental transfer (14). More than 60% of the human population experiences asymptomatic infection with the BK virus during their early years, after which, in individuals with competent immune systems, the virus enters a latent phase and remains in the urinary tract for the duration of life (12). Among immunosuppressed individuals, reactivation of the BK virus can precipitate significant medical issues, such as BKV-associated nephropathy in specific kidney transplant recipients (15). In certain recipients of allogeneic hematopoietic stem cell transplants, reactivation of the virus may lead to the development of hemorrhagic cystitis (13).

The oncogenic capacity of the BK virus to induce cellular transformation has been extensively validated through both in vitro and in vivo methodologies (6, 16). During latency, two viral oncoproteins contribute to this oncogenic potential: the large T antigen, which predominantly interacts with p53 family proteins and pRb tumor suppressor proteins, and the small t antigen, which exerts its transformative effect by inhibiting protein phosphatase 2A (PP2A), recognized as a tumor suppressor protein (6). Furthermore, BK virus has been acknowledged as a potential contributor or co-factor in the tumorigenesis of various malignancies, including head and neck cancers (7), brain tumors (17), prostate tumors (8), HIV-associated Salivary Gland Disease lymphomas (18), and bladder tumors (19).

The objective of this investigation was to examine the prevalence of the BK virus and its genotypes within colorectal cancer tissues and urine samples collected from patients undergoing surgical intervention at the medical facility.

MATERIALS AND METHODS

Study population. A cross-sectional investigation was conducted involving 50 diagnosed adenocarcinoma colorectal cancer (CRC) patients, comprised of 26 females and 24 males, who were referred to Apadana Hospital in Ahvaz for surgical intervention between February 2021 and March 2022. None of the participants had undergone neoadjuvant therapy prior to surgical procedures. In this study, three distinct specimen types were obtained from each participant: 1- fresh colorectal tumor biopsies, 2- paired adjacent tissues (non-cancerous tissue located 15-20 cm away from the tumor) collected by a surgeon following the excision of the cancerous organ, and 3- approximately 15-20 milliliters of urine samples were acquired from newly installed catheter and urine collection bags. Biopsies were promptly immersed in 400 µl of RNA stabilization buffer and subsequently frozen at -20°C. All pathological and histological evaluations of tumor and adjacent (non-cancerous) tissue biopsies were verified by a qualified pathologist. Urine samples were subjected to centrifugation at 1400g for 25 minutes, with the resulting precipitates immediately stored at -20°C.

DNA extraction from biopsies and urine samples. DNA was extracted from neoplastic tissues, adjacent non-tumorous tissues, and urine precipitates. The extraction process was conducted utilizing an internally developed phenol/chloroform protocol alongside salting out methodologies, as previously delineated (20). The quantification and assessment of the purity of the extracted DNA were conducted using a nanodrop spectrophotometer (Thermo Fisher, USA). The purified DNA samples were stored at -20ºC for future analysis. The validation of the extracted DNA was confirmed through the amplification of the human β-globin gene. All samples that tested positive subsequently underwent viral DNA amplification.

Viral DNA amplification and genotyping. Nested Polymerase Chain Reaction (PCR) was employed for

the detection of the BK virus VP1 region, utilizing an outer consensus primer pair VP1-7/VP1-2R to amplify a fragment of 579 bp, followed by the application of the inner primer pair 327-1/2 for the amplification of a 327 bp sequence (Table 1) (21). The genomic region spanning from 1630 to 1937 (327 bp) of the BK viruses was identified as a hypervariable region within the BK virus genome, which is critical for determining the genotypes of the BK virus (21).

The polymerase chain reaction (PCR) mixture consisted of 10 µL of a 2X master mix (Amplicon, Denmark), 1 µL (10 pmol/ µL) of each primer, 200-300 ng of DNA template, and distilled water to achieve a final volume of 25 µL. In each PCR assay, a microtube containing all PCR components, excluding the DNA template, was incorporated as a negative control, while a positive control featuring BK virus DNA was also included, employing the following thermal cycling protocol: an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 60 seconds, concluding with a final extension at 72°C for an additional 10 minutes. The subsequent round of amplification was performed using 1 µL of the product from the first round, along with 1 µL (10 pmol/µL) of each primer. 10 µL of PCR master mix (2x) and distilled water to a final volume of 25 µL. Thermal conditions were as followed: at 94°C for 5 minutes, 32 cycles at 94°C for 30 seconds, at 54°C for 30 seconds, and extension at 72°C for 60 seconds. The PCR product of 327 bp shows the positive result.

Gel electrophoresis. 2% agarose gel and a 100-bp DNA ladder were used to separate and detect PCR products in 20 minutes. Positive samples were subjected to Sanger sequencing.

Sequencing and phylogenic analyses of VP1. For the verification, the sequencing results of the partial "VP1" region of the isolates BK virus genome were aligned using the NCBI BK virus database. The isolated VP1 BK virus sequences were also aligned with the VP1 BK virus reference sequence by SnapGene software (version 3.2.1). In order to determine the BK virus genotyping, a phylogenetic tree was constructed by Maximum likelihood method for each isolated partial VP1 region of BK virus genome under the Kimura 2-parameter distance model with 1000 bootstrap replicates, scale bar used for this analysis was set at 0.01. The MEGA software version 6 was employed to implement these methods. The sequences of partial VP1 region of BK virus with their accession numbers, isolated from 8 tumors and 6 urine samples, were randomly selected and aligned with VP1 sequences of different BK virus genotypes isolated from various regions around the world (Fig. 1).

Statistical analysis. Data analysis was performed using SPSS software version 24.0. Chi-squared and/or Fisher's exact test were employed to analyze the data. The significance level was considered ≤ 0.05.

Ethical considerations. This study was conducted following receiving approval ethical code IR.AJUMS.REC.1401.400 by the ethics committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Prior to sample collection, informed consent was obtained from patients or their corresponding relatives.

RESULTS

Fifty freshly procured colorectal tumor specimens along with fifty corresponding adjacent tissues were subjected to investigation for the presence of viral DNA. The average age of the individuals enrolled in the study was 61 ± 12.4 years, with an age range spanning from 37 to 86 years. Nevertheless, there were no statistically significant differences observed in BK virus positivity among age groups. The quantity of positive samples for BK virus DNA in tumors and urines were not sensitive to gender as well.

The presence of BK virus DNA was confirmed in 15/50 (30%) colorectal tumor specimens and 3/50 (6%) adjacent tissues (p-value = 0.008). A total of 6/40 (15%) urine samples exhibited positive results for BK virus DNA, among which, three samples were concurrently positive for BK virus DNA in colorectal tumor tissue as well, while the remaining three were solely positive for BK virus DNA in urines. Randomly selected, 14 samples including 8 tumor and 6 urine samples positive for VP1 BK virus with accession numbers OR064035- OR064044 and OR113380 - OR113383 are recorded in GenBank. Detailed information regarding demographic and clinical characteristics, stratified by the presence or absence of BK virus DNA, are detailed in Fig. 1.

Within the subset of samples that successfully se-

Table 1. Primer sets used for BK virus detection

Primers	Sequence	Fragment size	Reference
VP1-7	5'-ATCAAAGAACTGCTCCTCAAT-3'	579 bp	(21)
VP1-2R	5'-GCACTCCCTGCATTTCCAAGGG-3'		
327-1	5'-CAAGTGCCAAAACTACTAAT-3'	327 bp	(21)
327-2	5'-TGCATGAAGGTTAAGCATGC-3'		

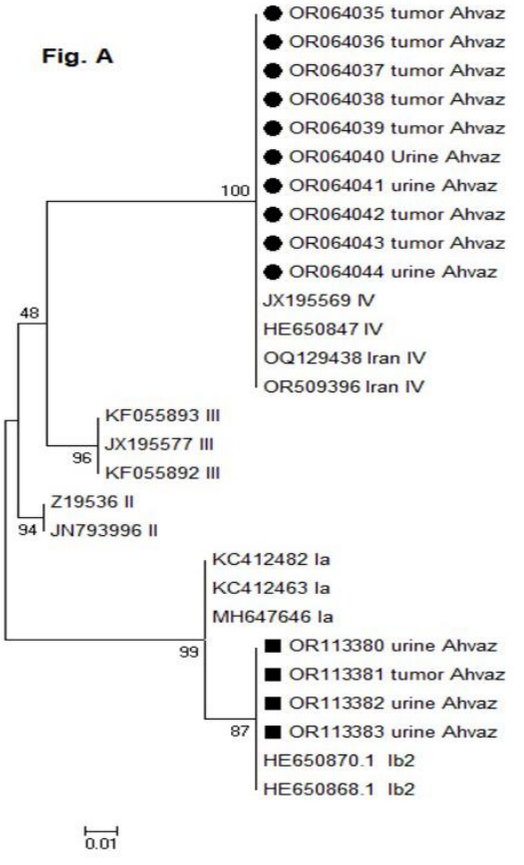

Fig. 1. The phylogenic tree was constructed by Maximum Likelihood Method for VP1 sequences BK virus genome isolates from CRC patients. The phylogenetic tree results indicated that the BK virus isolated from Ahvaz (OR064035-OR064044) clustered with genotype IV, JN794001.1 and MK647972.1 which were isolated in France. The BK virus isolated from Ahvaz (OR113380-OR113383) were cluster with BK genotype 1a MH647646, MH647646, MH647648, KC412462, KC412463 which were isolated from Brazil. An interesting thing was a male CRC patient tumor sample, OR064041 which identified as BK virus genotype 4, while his urine OR113383 was BKV genotype 1. The Tamura-Nei model was employed for the Maximum Likelihood method with 1000 bootstrap replicates. The scale bar was set at 0.01.

quenced for BK virus in tumors and urine, 9 tumor tissues (60%) and 3 urine samples (50%) were confirmed to be positive for BK virus type 4 (p-value < 0.001). Whereas, 2 tumor samples and 3 urine samples were attributed to BK virus type 1b2.

Assessing CRC risk factors including diabetes mellitus, smokers/drug users and clinical findings revealed no statistically significance related to BK virus status.

DISCUSSION

Colorectal cancer ranks as the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths, globally. These cancers are graded based on differentiation level. In present study, classic adenocarcinoma (88%) and well to moderately differentiated adenocarcinomas (90%) were the most identified cases (Table 2).

The BK virus as a known widespread infectious agent with a potential oncogenic nature, has been isolated from some different kinds of malignant tissues, alone or in accompany with other oncogenic viruses such as HPVs (11, 17, 22, 23). In present study, 15/50 (30%) of the CRC patients showed positive BK virus DNA in tumor tissue, which is in agreement with Jarzyński et al. in Poland reporting 30% BK virus DNA detection rate and Tseng et al. in Taiwan detected BK virus DNA in 36% of CRC specimens (11, 24), while Casini et al. from Italy reported 89% detection rate among the CRC samples (25). In contrast, Sarvari et al. failed to detect BK virus DNA in 210 CRC tissues samples, in Shiraz city of Iran (26). Khabaz et al, in Saudi Arabia also failed to isolate BK virus DNA in their 83 colorectal tissue specimens (27).

As part of this study, the sequencing was performed and the results revealed 9 tumor tissues and 3 urines as BK virus genotype 4. While 4 samples including 2 tumor tissues and 3 urine samples were exhibited to be BK virus genotype 1b2. The detection of BK virus genotype 4 and genotype 1 was appeared to

Table 2. Demographic information of samples regarding the BK virus status

Clinical or Pathologic feature	BK Positive (%)	BK Negative (%)	p-value
Tumor tissue=50	15 (30%)	35 (70%)	0.008
Normal adjacent tissue=50	3 (6%)	47 (94%)	
Age			
≥60=32	7 (22%)	25 (78%)	0.09
<60=18	8 (44%)	10 (56%)	
Gender			
Female=26	9 (35%)	17 (65%)	0.33
Male=24	6 (25%)	18 (75%)	
Health conditions			
DM=14	2 (14%)	12 (86%)	0.12
Non-DM=36	13 (36%)	33 (64%)	
Smoker/Drug user=10	4 (40%)	6 (60%)	0.34
Non-smoker/Non-drug user=40	11 (27%)	29 (73%)	
Tumor location			
Ascending colon=9	3 (33%)	6 (67%)	0.25
Descending colon=6	4 (67%)	2 (33%)	
Sigmoid=18	4 (22%)	14 (78%)	
Rectum=15	4 (27%)	11 (73%)	
Transverse colon=2	0 (0%)	2 (100%)	
Differentiation			
Well differentiated =25	9 (36%)	16 (64%)	0.28
Moderately differentiated =20	6 (30%)	14 (70%)	
Poorly differentiated =5	0 (0%)	5 (100%)	
Adenocarcinoma type			
Classic =44	12 (27%)	32 (73%)	0.26
Mucinous =5	3 (60%)	2 (40%)	
Signet ring =1	0 (0%)	1 (100%)	

DM, Diabetes mellitus.

be in agreement with BK virus genotypes global and regional distribution (28, 29). In this study we also found a CRC patient whose BK virus genotype tumor was 4 (OR064041) while his urine sample was genotype 1 (OR113383). This indicates the possible route of fecal-oral transmission of BK virus, however, it necessitates further investigations.

In this survey, high prevalence of BK virus DNA was detected among tumor tissue of CRC patients 15/50 (30%), when compared with the prevalence of 21/164 (12.8%) BK virus in normal population (30) it was found to be significant (P=0.004).

The NCCR region of BK virus consists of two forms, namely archetype (ww) and rearranged (rr) variants (31). Deletions and duplications within the NCCR sequence are a result of the viral DNA's continuous replication during reactivation, which leads to the emergence of rearranged NCCR and rearranged variant viruses. Previous research suggest the relation between rearranged NCCR variants and viral integration and tumorigenesis (31), indicating that investigating NCCR sequences could be beneficial for future research.

The presence of BK virus DNA has been well documented in sewage and surface water in Italy, Pakistan and Brazil (32-34). The BK virus detection has been also reported in drinking water in Nepal (35). The relative abundance of BK virus in different water sources and its persistence in urine, strengthen its potential to be transmitted via fecal-oral route. Some research have suggested, JC virus and BK virus investigation in sewage water can even be a useful tool for identifying human-sourced fecal pollution (36, 37).

Stool samples can be considered as non-invasive

tools for BK virus investigations in gastrointestinal complications and cancers. Given the probable role of BK virus in cancer development and its fecal-oral transmission, implementing preventive health plans could potentially reduce the spread of this virus in communities. Consequently, this may help mitigate the cancer burden on societies.

The limitations of this research encompass a need for a greater sample size, the quantification of BK virus DNA through real-time PCR, assessment of both archetype NCCR and rearranged (rr) variants in BK virus isolates, the expression of BK virus large T Antigen in tumor cells and BK virus DNA investigation in stool, all of which are essential for forthcoming investigations. The CRC stool/urine samples positive for BK virus DNA, are suggested to be treated by any of the anti-BK virus drugs monitoring the inhibition of BK virus reactivation.

The current strategy in BK virus infection in cancers is to reduce the degree of immunosuppressive drugs the patient take, to allow the immune system regain itself (6). A contemporary research has also indicated that prevailing investigations do not endorse the utilization of leflunomide, cidofovir, quinolones, or IVIGs, which have demonstrated inconsistent outcomes (38). It appears that a vaccine candidate targeting the BK virus is imperative as a preemptive strategy to mitigate the diseases and malignancies associated with the BK virus.

CONCLUSION

A high prevalence of BK virus (30%) has been observed in malignant tissues of patients with colorectal cancer. The dominant genotype of BK virus in this particular research was identified as genotype 4. It is advisable to conduct screening for BK virus DNA in stool samples of patients using PCR or real-time PCR, which can serve as a non-invasive diagnostic tool. In cases where colorectal cancer patients' stool or urine samples test positive for BK virus DNA, further molecular testing is recommended for potential anti-viral treatment.

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