Annals of Agricultural and Environmental Medicine 2017, Vol 24, No 3, 440–445
www.aaem.pl

ORIGINAL ARTICLE

Occurrence of BK Virus and Human Papilloma Virus in colorectal cancer

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Jarzyński A, Zając P, Żebrowski R, Boguszewska A, Polz-Dacewicz M. Occurrence of BK Virus and Human Papilloma Virus in colorectal cancer. Ann Agric Environ Med. 2017; 440–445. doi: 10.26444/aaem/74648

Abstract

Introduction and objective. Colorectal cancer is one of the most common cancers worldwide. In Poland, it is the second most common cancer, regardless of gender. The aim of study was to analyze the incidence of HPV and BKV in the tissue of colorectal cancer and to determine the relationship between the presence of these viruses and the development of this cancer.

Materials and method. The experiments were conducted using 50 colorectal cancer tissues collected from histological sections. The clinical material was embedded in paraffin blocks. Next, DNA extraction was performed. Isolates of colorectal cancer tissue were tested for the presence of HPV DNA. BKV DNA was detected by PCR using specific primers and then differentiated from JCV by digestion with *BamHI* enzyme.

Results. In clinical specimens taken from patients with colorectal cancer, HPV DNA was detected in 20% of cases. In 10% of cases the presence of HPV type 18 was confirmed, in the other 90% of the samples HPV type 16 was detected, while the presence of BKV was confirmed in 30% of cases. Coinfection with HPV and BKV was shown in 12% of patients. In one case, BK virus coexisted with HPV type 18, in the remaining 5 cases with HPV type 16.

Conclusions. Developing colorectal cancer can show no symptoms, even for many years. This is why it is so important to become familiar with as many etiological factors as possible. The development of many human neoplasms is often initiated by exposure to infectious agents – such as bacterial or viral infections. Similar to the human papillomavirus, the BK virus was detected in clinical specimens. It seems that HPV and BKV infections can contribute to the neoplastic process, which requires detailed studies on a larger group of patients.

Key words

BKV, HPV, colorectal cancer.

INTRODUCTION

Colorectal cancer is one of the most common cancers worldwide. In Poland, it is the second most common cancer, regardless of gender [1]. Colorectal cancer is one of the most common types of solid tumours occurring in adults [2]. The World Health Organization estimates that from 2030, the number of newly diagnosed cases of colorectal cancer will increase by 77%, and mortality by 80%. In the United States, one in four deaths is the result of cancer [3, 4]. Colorectal cancer is a tumour that occurs in the colon, the rectosigmoid junction, the rectum and the anus. In the majority of cases, colorectal cancer develops on the pedunculated, much less so in the non-pedunculated adenocarcinomas, whereby in the metaplastic processes a non-invasive cancer has developed only in the mucosa, and afterwards an invasive cancer beyond the lamina propria of the mucosa [5].

Developing colorectal cancer can show no symptoms, even for many years. That is why it is so important to recognize as many etiological factors as possible, in order to detect cancer at an early stage when there are still good chances for recovery [6].

The development of many human neoplasms is often initiated by exposure to infectious agents – such as bacterial or viral infections. Especially viruses are increasingly being seen as a cause of the development of the processes of carcinogenesis. Among the cancers occurring in the human population close to one-fifth it is connected with infection as a causative factor. HPVs cause a variety of benign, precancer or malignant neoplasms of the genital and anal areas, as well as head and neck cancers. Hepatitis B and C viruses may cause hepatocellular carcinoma, while Epstein-Barr virus causes Burkitt's lymphoma, HTLV-leukemia. Chronic bacterial Helicobacter pylori infection can lead to the development of gastric cancer [7, 8].

Human papillomavirus has a proven importance in the pathogenesis of cancers of various placements. HPV is built from a capsid deprived of a coat. Its genome is a double-stranded circular DNA molecule, which includes the regions: early and late, separated by a regulatory region. Proteins E6 and E7 play a key role in a neoplastic transformation. The combination of E6 with P53 proteins causes impairment of cell cycle regulation. E7 protein, by binding and inactivating proteins pRb, leads to loss of control of the cell cycle of an infected cell [9]. The data indicates that Human papillomavirus of the high-risk types 16 and 18, and low-risk types 6 and 11 are of major importance in the pathology connected with HPV infections 10]. Because polyoma viruses are common viruses in the population worldwide cause various infections of many mammalian species, including humans. In immunocompetent subjects, no clinically relevant infection occurs, their reactivation can only happen [11]. Primary infections are usually asymptomatic. It is estimated that more than 80% of the adult population have detectable antibodies

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Received: 13 September 2016; accepted: 25 January 2017; *first published: May 2017*

against polyomaviruses BK and JC [12]. After infection, the viruses persist in the cells for a lifetime. Infection with BK, KI, and WU viruses usually occur in early childhood, while JCV infection is observed several years later. Polyomaviruses are transmitted via respiratory and faecal-oral routes, and are also carried by blood and a transplanted organ. The tissue of the urinary tract is considered to be the main location of BKV and JCV persistence, while KIV and WUV persist in the tissues of the respiratory system [13].

The best-known viruses of this kind are BK and JC. JC virus can be detected in the nervous and brain tissue, tonsils, lymph nodes, colon, bone marrow, liver, spleen and in other tissues, including blood, plasma and lymphocytes. This virus is responsible for the occurrence of a progressive multifocal leukoencephalopathy, a disease causing demyelination of the central nervous system [14]. The BK virus may cause interstitial nephritis in a transplanted kidney and haemorrhagic cystitis. BKV may also contribute to the narrowing of the ureters, pneumonia, vascular diseases, retinopathy, inflammation of the brain, colorectal cancer and even organ failure [15, 16].

There are single reports confirming the participation of the virus in the development of urothelial malignancies and renal tubular tumours in patients after kidney transplantation. There are also reports of the detection of BKV DNA in other tumour types, e.g. neoplasms of the large intestine, pancreas, prostate, skin and brain [17, 18]. Interpretation of data concerning the influence of polyomaviruses infections on neoplasia is ambiguous; however, the International Agency for Cancer Research Monograph Working Group belonging to WHO classified BKV and JCV in the group of factors 2B as "possibly carcinogenic to humans" [19].

OBJECTIVES

The major aim of study was to analyze the incidence of HPV and BKV in the tissue of colorectal cancer and to determine the relationship between the presence of these viruses and the development of this cancer. The secondary aim was to evaluate the prevalence of mutations SNPs in the promoter region of the gene TP53 in intraoperative material. Mutations were detected in two places: mutation A / G at position -250 and mutation T / C at position -216 of the transcription start position.

MATERIALS AND METHOD

Characteristics of the population and research material. The research material consisted of paraffin sections of retrospectively chosen colorectal cancer tissue taken from histopathological specimens from 50 patients hospitalized in the Department of Surgery of the I Military Hospital in Lublin, diagnosed with colorectal cancer. In the study group, 38% of patients were women, while men were the 62%. The average age of the group was 68 years (women – 70, men – 67). Patients were divided into two age groups: 40–59 – 28%, and 60 and more – 72%. 60% of patients in the study were from urban areas, while 40% from rural areas. The study took into consideration the patients' use of drugs, alcohol and nicotine. Among all patients, only 2% admitted to regular use of alcohol. In the study group, 22% of patients had smoked or currently smoked cigarettes. Most of the diagnosed cases of colorectal cancer, 84% were in histological stage G2. In 14% of patients, stage G1 was described, and in 2% of patients stage G3.

Also of importance in the research was the original location of cancerous changes in the individual sections of the large intestine. In 24% of the respondents, cancer was diagnosed in the rectal segment; an additional 22% of patients were diagnosed with cancer in the sigmoid colon; 26% with cancer in a section of the colon; a further 26% in the caecum; whereas 2% were diagnosed with cancer in both the sigmoid colon and rectum.

Research methods. Prior to the DNA isolation stage, the research material was deparaffinized. The DNA isolation was performed with QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. In order to verify the quality of the obtained DNA and the presence of inhibitors of the PCR, a reaction for the human β-globin using the primers KM04 and PC03 was carried out.

Detection of HPV and BKV. For the detection of genetic material of HPV in the isolates from the cancer tissue of the large intestine, the commercial kit HPV INNO-LiPA (Innogenetics) was used. For each series of tests positive and negative controls were included. The reaction was performed in the thermocycler Tpersonal (Biometra).

Reaction parameters:

94 °C 10 minutes – decontamination
94 °C 9 minutes – UNG inactivation and activation of the polymerase Hot Start
94 °C 30 seconds
52 °C 45 seconds
72 °C 45 seconds
} 45 cycles
72 °C 15 minutes – final extension.

After the finished amplification, the samples were applied on 2.5% agarose gel and the presence of product was checked. Positive samples were genotyped using the hybridization method according to the method suggested by the kit manufacturer.

Detection of genetic material of the BK virus. Performed through a PCR using specific primers. Due to the high homology of the genomes of the BK and JC viruses (75%) the primers are complementary to the DNA of both viruses. The primers' sequence is shown in Table 1. The composition of the reaction mixture for the amplification of the DNA of the polyomaviruses BK and JC is shown in Table 2. The reaction used 5 µl of DNA isolated from paraffin sections. For each series of tests, positive and negative controls were included. For positive control, a virus from the cell culture ATCC (VR-837) was used, the negative control used nucleases-free water. The reaction was carried out in the thermocycler Tpersonal (Biometra).

Reaction parameters:

95 °C 15 minutes – initial activation of Hot Start polymerase
94 °C 1 minute
55 °C 1 minute
72 °C 1 minute
} 40 cycles
72 ° C 10 minutes – final extension.

The presence of PCR product was checked on 2% agarose gel.

Table 1. Primer sequences specific for viruses BK and JC

Designation	Sequences	Size of the product
PEP1	5′ – AGTCTTTAGGGTCTTCTACC – 3′	176 bp for BKV 173 bp for JCV
PEP2	5′ – GGTGCCAACCTATGGAACAG – 3′	

Table 2. Composition of the reaction mixture for DNA BKV and JCV

Volume	Reagent
2 µl	10x PCR Buffer
4 µl	5xQ-Solution
0.4 µl	MgCl2
0.4 µl	dNTPs
0.5 µl	PEP1 (10mM)
0.5 µl	PEP2 (10mM)
0.1 µl	polymerase HotStart
make up to 15 µl	nucleases water free

The sequences of the PCR products vary depending on the virus and can therefore be differentiated using restriction enzymes. Samples with a positive result of the JC / BK virus amplification were cut with the restriction enzyme BamHI (Fermentas), in volume presented in Table 3. As a result of the cutting, the product size of BKV does not change, and in the case of the JC virus, the cutting results in two products with lengths of 120 bp and 56 bp.

Table 3. Composition of reaction mixture for BamHI enzyme

Volume	Reagent
17 µl	nucleases water free
2 µl	10xFast Digest Buffer
10 µl	PCR product
1 µl	BamHI enzyme

Examination of single nucleotide mutation (SNP) in gene TP53 promoter. In order to determine the frequency of SNP A -> G mutations at position -250, an amplification of a fragment of the TP53 gene promoter measuring 468 bp was carried out, using appropriate primers (Tab. 4). The composition of the reaction mixture in accordance with Table 5. The reaction was carried out in a thermocycler SensoQuest LabCycler under the following conditions: initial denaturation at 94 °C – 4 min, 45 cycles: denaturation at 94 °C – 40 sec, annealing 50 °C – 40 sec., elongation 72 °C – 1 min., and final extension at 72 °C – 10 min.

Table 4. Primer sequences specific for gene promoter fragment *TP53*

Designation	Sequences
TP53-F	5′- GAT ATT ACG GAA AGC CTT C – 3′
TP53-R	5′- AGC CCG AAC GCA AAG TGT – 3′

Analysis of the presence of an SNP at positions -250 and -216. Carried out by restriction digest of resulting PCR products with two enzymes in two separate reactions (Tab. 6), each in a volume of 30 µl. The enzyme BglI (FastDigest BglI, Thermo Scientific) recognizes and digests a specific sequence, which appears in the amplified fragment of the promoter of the gene TP53 once, and only in the event of an SNP A/G mutation occurring at position -250, while BfaI (FastDigest BfaI, Thermo Scientific) recognizes and digests a specific sequence twice in the event of an SNP T/C mutation occuring at position -216.

Table 5. Composition of reaction mixture for promoter fragment *TP53*

Reagent	Volume	Final concentration
Reaction buffer 10x (1,5 mM MgCl2)	3 µl	2 mM MgCl2
MgCl2	0.6 µl	
dNTP	0.6 µl	200 µM
Primer TP53-F	0.45 µl	0.15 µM
Primer TP53-R	0.45 µl	0.15 µM
Polymerase *Taq* (Qiagen)	0.15 µl	0.75 U
H2O	20.25 µl	-
DNA	4.5 µl	-

Table 6. Composition of the reaction mixture for BglI and BfaI enzymes

H2O	17 µl
Buffer 10 x FastDigest	2 µl
Restriction enzymes	1 µl
DNA	10 µl

Reaction products were separated by agarose gel electrophoresis. After digestion of enzyme BglI, the absence of mutations was seen as one band measuring in length 468 bp. If a mutation occured in the tested samples, two bands measuring 153 bp and 315 bp could be observed. In the case of enzyme BfaI, bands measuring 61 bp, 189 bp and 218 bp meant that there was a T -> C mutation at position -216. In the absence of mutations, two bands measuring 61 bp and 407 bp (Tab. 7) could be observed on the gel.

Table 7. Sites of occurence of TP53 promoter mutations. sequences recognized by the enzymes and length of products received form digestion

SNP	Restriction enzymes	Recognized sequence	Type alleles (number of sites of cutting)	DNA fragment (bp)
-250 A/G	BglI	GCC(N)4^NGGC	Wild (0) Mutant (1)	468 153. 315
-216 T/C	BfaI	C^TAG	Wild (1) Mutant (2)	61. 407 61. 189. 218

RESULTS

Presence of HPV and BKV in the tested clinical material. In clinical specimens taken from patients with colorectal cancer, HPV DNA was detected in 20% of cases. In 10% of cases, the presence of HPV type 18 was confirmed, in the other 90% of the samples HPV type 16 was detected, while the presence of BKV was confirmed in 30% of cases.

Coinfection with HPV and BKV was shown in 12% of patients. In one case, BK virus coexisted with HPV type 18, in the remaining 5 cases with HPV type 16.

HPV and BKV were detected more frequently in men. Among the HPV positive patients, 60% were males and 40% females. Similarly, among those BKV positive 67% were males,

33% females. Both viruses were most frequently detected in the age group above 60 years of age, HPV – 60%, and BKV – 67%. Meanwhile, in the age range of 40–59, HPV was detected in 40% and BKV in 33% of patients. The incidence of the virus does not depend on the place of residence. HPV was detected in 50% of urban and in 50% of rural residents. In patients infected with BKV, a slightly larger percentage of patients were those residing in rural areas – 60%, while urban inhabitants were the remaining 40%. Data obtained in interviews with patients showed that 10 patients HPV (+) and 33% of patients BKV (+) were smokers, while 10% and 7% (Tab. 8) excessively consumed alcohol.

Table 8. Socio-demographic data and incidence of HPV and BKV

	HPV (+). N=10		BKV (+). N=15	
Gender	Number – N	%	Number – N	%
Females	4	40%	5	33%
Males	6	60%	10	67%
p – probability	0.8841		0.6562	
Age	number – N	%	number – N	%
40–59 years	4	40%	5	33%
60 years and more	6	60%	10	67%
p – probability	0.3447		0.5824	
Residency	number – N	%	number – N	%
City	5	50%	6	40%
Village	5	50%	9	60%
p – probability	0.4704		0.0587	
Smoking	number – N	%	number – N	%
Yes	1	10%	5	33%
No	9	90%	10	67%
p – probability	0.3057		0.2053	
Alcohol consumption	number – N	%	number – N	%
Yes	1	10%	1	7%
No	9	90%	14	93%
p – probability	0.0433*		0.1228	

* statistically significant data

Clinical data and occurrence of viruses. The prevalence of the examined viruses in each section of the large intestine was analyzed (Tab. 9). Among the patients infected with HPV, in 3 of them the virus was detected in the caecum (30%), in 3 patients in the rectum (30%), in 2 patients in the ascending, the transverse and the descending colon (20%), and in 2 patients in the sigmoid colon (20%). In patients infected with BKV, in 6 of them the virus was detected in the caecum (40%), in 4 patients in the colon (26.5%), in 4 patients in the sigmoid colon (26.5%), and in one patient in the rectum (7%).

Table 9. Clinical data and the frequency of occurrence of HPV and BKV

	HPV (+) N=10		BKV (+) N=15	
Location	Number – N	p – probability	Number – N	p – probability
Caecum	3	0.7471	6	0.1395
Ascending colon. Desceding colon	2	0.6286	4	0.9439
Sigmoid	2	0.7405	4	0.7725
Rectum	3	0.7471	1	0.0413*
Stage	Number – N	p – probability	Number – N	p – probability
G1	0	0.6135	0	0.5084
G2	9	0.5628	10	0.0286*
G3	1	0.6835	5	0.0099*

* statistically significant data

Among the patients from the study group who were diagnosed with HPV as well as BKV, no tumours with a low grade of histological differentiation – grade G1, were found. In the case of 9 patients HPV (+) (90%) and 10 BKV (+) (67%), the tested material came from tumours having an average grade of histological differentiation – G2. In the case of one patient, HPV (+) (10%) and 5 BKV (+) (33%) the tested material came from cancer with a high grade of histological differentiation – G3.

SNP mutation detection in the TP53 gene promoter. In samples from patients in the study group, no SNP mutations in the TP53 gene promoter at position -250 A/G or at position -216 T/C were detected.

DISCUSSION

Neoplastic diseases are among the most important problems of modern medicine. Global data shows that in men, in terms of incidence, colorectal cancer ranks as the third after lung and bronchus cancer and prostate cancer, with approx. 640,600 cases per year, whereas in women it occupies the second place immediately after breast cancer, with approx. 570,100 cases per year [20].

In connection with the growing number of cases of colorectal cancer, far-reaching prevention is important, including the identification of risk factors of developing this cancer. It is known that the process of oncogenesis that conditions the development of colorectal cancer is multifactorial. The role of infectious agents in the process of carcinogenesis of colorectal cancer was, and is, the subject of numerous studies but remains undetermined. Infectious agents, especially viral infections, are increasingly often seen and recognized as an essential component responsible for carcinogenesis [21].

The development of many human cancers is initiated by exposure to infectious agents, including viruses. The human papillomavirus – HPV is the most thoroughly recognized virus with proven oncogenic potential. The human papillomavirus exhibits dermotropism, and numerous studies have confirmed a close correlation between infection with this virus, and the development of cervical epithelial neoplasia and neoplasia of other parts of the feminine urogenital tract [22]. Furthermore, there are several results suggesting an active role of HPV in other cancers, e.g. breast, esophagus or respiratory tract cancer [23, 24].

The HPV infections' impact on carcinogenesis in the gastrointestinal tract has been proved in cases involving its end section – the anus. The process of carcinogenesis begins usually within the transition zone between the flat epithelium of the anal canal and the glandular epithelium of the rectum [25].

The data suggesting an impact of an HPV infection on the development of colorectal cancer appeared in 1990. Immunohistochemical research carried out by the Kirgan

et al. has proved the presence of viral antigens in the material derived from the large intestine tissue fragments. HPV was detected in 23% of samples of healthy colon epithelium, in 60% of adenomas and in 97% in the tissue derived from colorectal cancer samples [26].

According to the publication of Damin et al. [27], which is a meta-analysis of 16 research studies published between 1990–2012, in which HPV has been detected in colorectal cancer in different regions of the world, the prevalence of the virus fluctuates from 0–84.2% with the mean value of 38.5%. The total number of patients included in the analysis was 1,436, with the mean value of 91.4 patient per a single published study.

HPV 18 was the most commonly detected HPV type in the cancerous tissues of the large intestine, with values from 52.85% – 95% of cases. HPV 16, the second in incidence, was present in from 32.85% – 95% of cases. HPV type 33 was third in terms of the number of cases, ranging between 17.07% – 95% of them. In Europe, the most frequently detected type of virus was HPV 18, present in 73.34% of cases [27]. No connection between HPV and colon cancer has been demonstrated in other studies, e.g. Aghakhani A. et al. [28] and Taherian H. et al. [29]. In this study, research of colorectal cancer tissue has demonstrated the presence of HPV DNA in 20% of the samples. This value is below the average worldwide occurrence of this virus (38.5%), but is higher than the European average (7.7%). Significant differences were observed in the detection rate of certain types of human papillomavirus, HPV type 18 was present in 2% only, while HPV type 16 in 18% of the examined patients. Of all the human polyomaviruses BKV and JCV are the most commonly described in the population. Serological studies indicate that more than 80% of people are infected with these viruses worldwide [30]. Analysis of the samples revealed the presence of BKV virus in 30% of cases. This number is difficult to interpret, because different scientific publications indicate the considerable differences in the polyomaviruses detection. In the study by Giuliani et al. [31], which analyzes the occurrence of BKV in colorectal cancer with the PCR method, the infection therein concerns only 9% of patients. In turn, Casini et al. [32] indicate the presence of BKV DNA in 88.9% of the samples taken from pieces of colorectal cancer. In both cases, as in the presented study, the same method was used for detection of BKV, i.e. PCR with specific primers, followed by digestion with restriction enzyme BamHI. Differences in the frequency of the virus detection may be due to the size of the study group in the above-mentioned publications, population genetic variation, or differences in the socio-demographics.

The BK virus is a threat mainly for transplant patients, immunosuppressed patients or those with AIDS. Meanwhile, Fiorina et al. [33] did not find the presence of any oncogenic viruses, including BKV in colorectal cancer. This confirms the great importance of environmental and social factors in the development of this type of cancer.

The histological differentiation grading of a tumour is a prognostic factor. It is believed that tumours with lower differentiation are characterized with a more aggressive course. In the currrent study, it was found that among patients with cancer of the G1 grade of differentiation there was not a single one with an HPV infection. No studies evaluating the correlation between an HPV infection and the degree of histological differentiation could be found in the literature available. Whereas the reports on this subject in patients with other types of cancer are inconclusive. Dahlgren et al. [34] and Klussmann et al. [35] found that HPV infection is more common in cancers of lower differentiation, while Mellin et al. [36], and Kay et al. [37] did not confirm such a correlation.

A separate part of the study concerned the occurrence of SNP mutations in two locations of the human TP53 gene promoter. The presented study is of an innovative nature, as there are no scientific articles in which the presence of SNP mutations at position -216 and -250 in the TP53 gene promoter in material derived from colorectal cancer was detected. However, similar studies on other material were carried by Hsieh Y-Y. et al. [38] in 2007. They analyzed, using the same method as that used by the author of the current study, the presence of the above-mentioned mutations in samples from women diagnosed with uterine fibroids. The presence of SNP transversion mutation T/C at position -216 was confirmed by the results of these studies in 5.63% of cases, whereas an SNP A/G transversion change at position -250 concerned 6.88% of the people examined. In this study, neither SNP mutation at position -216, nor at -250, was observed in samples originating from patients with colorectal cancer. It seems that the development of colorectal cancer cannot be attributed to TP53 promoter mutations in the above-mentioned positions. HPV and BKV infections also do not cause the occurrence of SNP mutations in the examined area of this gene. It seems that HPV and BKV infections can contribute to the neoplastic process, which requires detailed studies on a larger group of patients.

CONCLUSIONS

The human papillomavirus was detected in 20% of patients with colorectal cancer, 90% of which was type 16, the remaining 10% was type 18, while the BK virus was found in 30%. Patients diagnosed with an HPV and BKV coinfection constituted 12%. In one case, the BKV virus coexisted with HPV type 18, in 5 cases with HPV type 16. Both viruses most frequently were detected in men aged over 60–60% of HPV and 67% BKV. Infection with HPV or BKV was usually determined in the tumour samples of a medium histologic degree of differentiation. No viruses were detected in samples from cancer with a low degree of histological differentiation. In the clinical material examined, no SNP mutations were detected. Locations in which the mutations' incidence was evaluated, may not be directly responsible for the formation of colorectal cancer

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