Assessment of prevalence of HPV DNA in colorectal adenocarcinoma in patients from the Podlasie region

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ABSTRACT

Purpose: The aim of the study was to assess the prevalence of human papillomavirus (HPV) DNA in colorectal adenocarcinoma in patients from the Podlasie region undergoing surgery for the tumor.
Materials and methods: We examined 40 solid colorectal tumors taken during surgical treatment at the 2nd Department of General and Gastrointestinal Surgery, Medical University of Białystok. HPV was detected by means of polymerase chain reaction (PCR) and *in situ* hybridization (ISH). The tests were performed on formalin-fixed, paraffin-embedded tumor tissue. Two pairs of primers were used for the detection of HPV DNA by PCR. Pair pU-1M/pU-2R enables detection and identification of high-risk HPV (HPV 16, 18, 31, 33, 35, 52b, 58), while pair pU-31B/pU-2R enables detection and identification of low-risk HPV (HPV 6, 11). The ISH was performed with the use of biotin-labelled dsDNA probes, using Wide Spectrum HPV DNA Probe Cocktail Biotinylated kit, DAKO Cytomation.
Results: HPV DNA was found in 21 (52.5%) of the examined colorectal tumors. The PCR revealed the presence of viral DNA in 19 (47.5%) tumors. The ISH revealed the presence of HPV DNA in 16 (40%) of the examined tumors.
Conclusion: The findings of this study correlate with similar results conducted by other research groups. However, this is the first study of colorectal tumor samples taken from patients of the Podlasie region. Therefore, the association between environmental factors, HPV infection, and tumor stage should also be verified in a larger study population. Further studies confirming the presence of HPV DNA in colorectal tumor tissue in populations from different regions of Poland are needed.
Key words: Human papillomavirus, colorectal adenocarcinoma, polymerase chain reaction, *In situ* hybridization

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INTRODUCTION

Colorectal carcinoma becomes common gastrointestinal malignancy. It provides second cause of morbidity and mortality among both men and women. In Poland, colorectal carcinoma is diagnosed in approx. 11,000 individuals annually, and about 8,000 patients die of this tumor. Approximately 45% of tumors are located in the rectum, and histopathologically 95% of them are adenocarcinomas.

Proposed risk factors include environmental factors, mostly poor diet (rich in fat and carbohydrates and poor fibers); genetic predispositions related to corrupted DNA repair genes and APC gene mutations, as well as intrinsic factors, such as adenocarcinoma or ulcerative colitis. At the end of 20th century, certain role of oncogenic DNA viruses, mostly EBV and HPV in colorectal carcinogenic was postulated [1].

HPV may be detected in many human neoplasms, both malignant and benign ones. It may be found in tumors of skin, urinary tract, respiratory system, head and neck, first of all, of female reproductive system [2]. Oncogenic properties of HPV result from the presence of viral oncoproteins E6 and E7, which action consist of disorganization of cellular cycle in the infected cell and the ability of the virus DNA to integrate with cellular genome resulting in the genome destabilization and uncontrolled proliferation [2].

According to the studies concerning the incidence of HPV in colorectal carcinoma published worldwide to date, the virus is detected in 0 to 83% of cases [3-24].

The aim of the study was to assess the prevalence of human papillomavirus (HPV) DNA in colorectal adenocarcinoma in patients from the Podlasie region undergoing surgery for the tumor.

MATERIAL AND METHODS

The authors selected 40 solid colorectal tumors taken during surgical treatment in 2nd Department of General and Gastrointestinal Surgery, Medical University of Bialystok. The tumors were taken from patients aged 27-88 years (19 women and 21 men). The location of the tumors were as following: ascending colon (7 cases), descending colon (5 cases), sigmoid colon (10 cases), rectum (14 cases), transverse colon (2 cases) and cecum (2 cases). Detailed characteristic of patients and tumor are presented in Table 1.

HPV was detected by means of PCR and ISH. The tests were performed on formalin-fixed, paraffin-embedded tumor tissue. DNA was isolated by means of a kit for DNA isolation from tissues stored in the form of paraffin blocks (QIAamp DNA FFPE tissue kit, QIAGENE). Before the isolation, 10 μm-thick sections were placed on a slide and deparaffinised. Then the material was scraped with a sterile needle and transferred to eppendorf tubes. Further procedure was based on the isolation protocol provided by the kit manufacturer. Negative control involved the use of the reagents alone. To verify the quality and ability of amplification of isolated DNA, exon 1 of K-ras gene was amplified. Two pairs of primers were used for the detection of HPV DNA. Pair pU-1M/pU-2R enables detection and identification of high-risk HPV (HPV 16, 18, 31, 33, 35, 52b, 58) while pair pU-31B/pU-2R enables detection and identification of low risk HPV (HPV 6, 11). The primers are located within E6 and E7 genes [25]. The specific products of PCR reaction (size 228-267 bp) were identified in ethidium bromide-stained agarose gel.

In situ hybridization (ISH) was performed with the use of biotin-labelled dsDNA probes-Wide Spectrum HPV DNA Probe Coctail Biotinylated kit, DAKO Cytomation (ref.no. Y1404). The kit enables detection of several most common mucosal types, i.e. HPV 6,11,16,18,31,33,35,45 and 51. Probes bound to HPV DNA were visualized by means of DAKO Cytomation's kit Gen Point Catalyzed Signal Amplification System for in situ hybridization (ref.no.K0620). Hybridization was performed according to guidelines of the kit manufacturer: Modification to Gen Point procedure for DAKO Cytomation Human Papillomavirus (HPV) DNA probes. Negative and positive controls consisted of CeSki cells containing HPV 16. Two sections of the tumor examined were put on each

Table 1. Clinical and pathological features of 40 colorectal adenocarcinoma patients

Parameters	Number of patients	Percentage
Gender		
Female	19	47.5
Male	21	52.5
Age (yr)		
< 55	8	20
> 55	31	77.5
unknown	1	2.5
Tumor site		
Proximal(c,a,t)	9	22.5
Distal (d,s,r)	31	77.5
Tumor differentiation		
Moderately (G2)	36	90
Poorly (G3)	2	5
unknown	2	5
Tumor stage		
I	8	20
II	11	27.5
III	8	20
IV	11	27.5
unknown	2	5

c- cecum, a- ascending colon, t- transverse colon, d-descending colon, s- sigmoid colon, r -rectum

slide; DNA probe was applied on one section, while distilled water was applied on the other one. The study was approved by the Ethical Committee of the Medical University of Bialystok, Poland.

Statistics were calculated using Statistica 7.1, StatSoft, Cracow, Poland. The chi-square test was applied in this study. Statistical significance was defined as $P < 0.05$.

RESULTS

HPV DNA was found in 21 (52.5%) of examined colorectal tumors. PCR revealed the presence of viral DNA in 19 (47.5%) tumors, including 12 (30%) cases of low-risk HPV DNA, 4 (10%) cases of high-risk HPV DNA and 3 (7.5%) cases of co-infection with high- and low-risk HPV. ISH revealed the presence of HPV DNA in 16 (40%) of examined tumors. Positive hybridization signal in the form of brown deposits was found within the cell nucleus. In 14 (35%) cases, HPV DNA was detected by both PCR and ISH, in 5 (12.4%) cases by PCR only and in 2 (5%) cases only by ISH. HPV was found in 7 rectal tumors, 7 sigmoid tumors, 4 tumors of the ascending colon, and 1 tumor of the descending colon, transverse colon and cecum each. HPV was detected in 12 women and 9 men. Characteristic of HPV positive patients is presented in Table 2. No significant relationships (NS) between the virus's presence and age, gender or tumor location were found. Relationship between HPV infection and parameters of adenocarcinoma tumor is presented in Table 3.

Pt- patients, TD, - tumor differentiation, u/k- unknown," -" non detected, "+" detected

Table 2. HPV positive patients characteristics

Pt.	Sex	Age	TD	TNM	HPV DNA		
					PCR		ISH
					LR	HR	
1	M	57	G2	II	+	-	-
2	M	47	G2	I	-	+	+
3	F	84	G2	IV	-	-	+
4	F	72	G2	IV	+	-	+
5	F	67	G2	III	+	+	+
6	M	69	G2	IV	+	-	+
7	F	88	G2	III	+	-	+
8	F	37	G2	II	+	-	+
9	M	76	G2	I	+	-	+
10	F	75	G2	II	+	-	+
11	F	79	G2	IV	+	-	+
12	F	75	G2	IV	+	-	+
13	F	72	G2	II	+	-	+
14	M	84	G2	I	+	-	+
15	F	73	G2	II	+	-	+
16	F	52	G2	III	+	+	-
17	M	77	G2	I	+	+	-
18	F	84	G3	II	-	+	-
19	M	63	G2	IV	-	+	+
20	M	u/k	G2	II	-	+	-
21	M	51	u/k	-	-	-	+

Table 3. Relationship between HPV infection and parameters of colorectal adenocarcinoma patient

Parameters	HPV	
	negative n (%)	positive n (%)
Gender		
Female	7 (37)	12 (63)
Male	12 (57)	9 (43)
Age (yr)		
< 55	4 (50)	4 (50)
> 55	15(48)	16 (52)
unknown	0	1 (100)
Tumor site		
Proximal(c,a,t)	3 (33)	6 (66)
Distal (d,s,r)	16 (52)	15 (48)
Tumor differentiation		
Moderately (G2)	17 (47)	19(53)
Poorly (G3)	1 (50)	1 (50)
unknown	1 (50)	1 (50)
Tumor stage		
I	4 (50)	4 (50)
II	4(36)	7 (64)
III	5 (62.5)	3 (37.5)
IV	5 (45.5)	6 (54.5)
unknown	1 (50)	1 (50)

c- cecum, a- ascending colon, t- transverse colon, d- descending colon, s- sigmoid colon, r -rectum

DISCUSSION

The presence of HPV is commonly detected in human tumors. In the current study, we found the presence of HPV in 52.5% of examined colorectal tumors. Studies conducted by Młynarczyk et al. [21] with a different group of Polish patients with carcinoma revealed the presence of HPV DNA in 77% of the cases. Comparable or higher ratio of HPV positive tumors was demonstrated by Cheng et al. [16,17], Buyru et al. [22], Damin et al. [18], Salepci et al. [23], Perez et al. [19,20]. Lee et al. [24] determining the virus presence in 52-84% of cases accordingly.

Unlike other studies, the researchers conducted by Mc Gregor et al. [12], Perez et al. [14], Yu et al. [10], Liu et al. [11], Deschoolmeester et al. [9], show the lower percentage of HPV positive tumors, 14-44% of cases accordingly. Although, several research groups, such as Boguszakova et al. [1], Cavatorta et al. [8], Dong et al. [3], Gornick et al. [4] and Audeau et al. [5] did

not find HPV DNA in the intestinal tumors. In general, the distribution and prevalence of HPV genotypes depend on the geographic region and demographic factors. High incidence of HPV in colorectal carcinomas is found in Latin America (64-74%), Taiwan (84%), Turkey (82%) [19,20,23,24], while lower incidence is observed in China (29%) and Belgium (14%) [9-11]. The summary of published cases worldwide of HPV DNA in colorectal adenocarcinoma is presented in Table 4.

Table 4. Summary of published cases of HPV DNA in colorectal adenocarcinoma

Country	**Autor**	**Number of cases**	**Method**	**% HPV positive**
USA	Kirgan 1990	30	ICC	97
			ISH	43
	Mc Gregor 1993	38	PCR-L1	32
	Bodaghi 2006	55	PCR-MY/GP + TS	51
	Gornick 2010	73	PCR-GP INNO-Lipa SPF10	0
Agrentina	Pérez 2005, 2006	54	PCR-MY/GP, SSCP	74
	Pérez 2010	75	PCR-MY/GP + TS 16, 18	44
Brazil	Damin 2007	72	PCR-MY/GP GP/GP	63.9
China	Yu 2002	32	PCR-TS 16,18	21.9
	Dong 2009	5	ISH	0
	Liu 2011	96	PCR-PGMY/ GP	29.2
Taiwan	Lee 2001	19	PCR –E6/E7 18	84
	Cheng 1995	70	PCR-E6/E7	52.9
Israel	Gornick 2010	106	PCR-GP, INNO-Lipa SPF10	0
New Zealand	Audeau 2002	20	ICC	0
Belgium	Deschoolmeester 2010	232	PCR-SPF10, INNO-Lipa	14
Spain	Gornick 2010	100	PCR-GP, INNO-Lipa SPF10	0
Turkey	Buyru 2006	53	PCR-MY	81
	Salpeci 2009	56	PCR –TS 16,18,33, 6, 11	82.1
	Yavuzer 2011	106	PCR-MY/GP	0
Germany	Sotlar 2001	4	PCR, ISH	0
Poland	Młynarczyk 2009	9	PCR-TS 6/11, 16/18	77
	Present study	40	PCR-E6/E7	47.5
			ISH	40

ICC – immunohistochemical system, ISH- *in situ* hybridization, PCR-L1 – PCR with primers to L1 region of viral genome, PCR-MY – PCR with MY09/My11 primers, PCR-GP – PCR with GP5+/GP6+ primers, PCR-TS – PCR with type-specific primers for L1 region or E6/E7 region of viral genome, INNO-Lipa SPF10 – genotyping system for HPV based on PCR with SPF10 primers and hybridization

The presence of HPV is commonly detected by PCR and rarely by ISH. In the current study PCR revealed the existence of low-risk HPV in 30% of the cases, high-risk HPV in 10% of the cases and of co-infection with high- and low-risk HPV in 7.5% of the cases. However, in situ hybridization confirmed HPV DNA in 40% of the tumors. In the present study, only tumor tissue was analyzed, similar to research conducted by Perez et al. [20] and Deschoolmeester et al. [9]. However, some authors also examine the incidence of HPV in both tumors itself and healthy adjacent tissue (within the distance 10-30 cm) [15,21-23] or healthy intestinal mucosa taken from tumor-free patients [11,26].

In tumor-adjacent tissue, HPV DNA is found in about 30% of cases [15,21-23] and in healthy intestinal mucosa in 0-23% of cases

[11,26]. In our studies, there was no possibility to analyze healthy, tumor-free intestinal mucosa. The most common (95%) histopathological form of colorectal carcinoma is adenocarcinoma , remaining 5% include squamous cell carcinoma (SCC), mixed carcinoma (adenocarcinoma +SCC) and undifferentiated carcinoma. Presence of HPV was detected also in colorectal squamous cell carcinoma [6,27-29]. Bognar et al. [27] detected HPV16 in sigmoid squamous cell tumor in 94-year-old women. HPV 16 was also present in 4 of 9 adjacent removed lymph nodes; in two of HPV-positive nodes metastases were found. Kong et al. [28] described 3 cases of squamous cell carcinoma (2 invasive rectal tumors, 1 colonic tumor in situ), in which HPV 16 was also detected. The tumors were collected from women aged 48, 53 and 49 years. Matsuda et al. [29] presented a case of 55-year-old, HIV-positive man, in whom presence of HPV was determined in rectal squamous cell tumor. Also, Sotlar et al. [6] confirmed presence of HPV in squamous cell carcinoma in 87-year-old man. Moreover, presence of mRNA of viral oncoproteins E6 and E7 was found in tumor tissue and healthy regular intestinal mucosa.

Presence of HPV DNA in colorectal tumors, as well as in regular intestinal mucosa has been proven by many authors; however, the role of HPV in carcinogenesis still arouses numerous controversies and reservations [4]. Currently, most attention is given to the assessment of incidence of HPV DNA in colorectal adenocarcinoma only. Moreover, research includes analysis of a possible relationship between the presence of HPV and expression of cellular genes involved in carcinogenesis, such as p53, K-ras, C-myc, FITH [10,19-21,30,31]. To date, only Weinberger et al. have assessed the level of HPV oncoprotein in colorectal adenocarcinoma. They analyzed a sample of 447 tumors and demonstrated significant relationship between the expression of HPV16 E6 oncoprotein and distal location of the tumor and stage of its advancement [32].

CONCLUSIONS

Our study shows the presence of HPV DNA in more than 50% of colorectal tumor samples. The findings of this study correlate with similar results conducted by other research groups [13,15-18]. Clarification of the role and extent, to which HPV contribute to the development of colorectal carcinoma requires further research on the expression of E6 and E7 oncoproteins. Also, there is variability in sensitivity and specificity between various methods for HPV DNA detection. Therefore, standardization of research for ensuring quality of the tests is needed. Further studies confirming the presence of HPV DNA in colorectal tumor tissue in populations from different regions of Poland are needed. Therefore, the association between environmental factors, HPV infection and tumor stage should be also verified in a larger study population.

Conflicts of interest

We declare that we have no conflicts of interest.

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