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CORRESPONDENCE

Detection of Human Papillomavirus Type 16 in Squamous Cell Carcinoma of the Colon and Its Lymph Node Metastases with PCR and Southern Blot Hybridization

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Abstract The etiological role of human papillomavirus (HPV) in a number of squamous malignant tumors is well known. Squamous cell carcinoma (SCC) of colon is a rare disease with uncertain etiology. Our objective was to detect possible HPV infection in a colon SCC patient. The 94-year-old female patient was operated due to colon tumor causing passage disturbances. Histology confirmed SCC. Tumor tissue and the removed lymph nodes were examined with polymerase chain reaction and Southern blot hybridization techniques. Of HPV types most often occurring in malignant tumors (16, 18) the presence of HPV type 16 could be confirmed in the primary tumor and in four out of the nine surrounding lymph nodes, of which two were metastatic. HPV-16 infection could be detected in an SCC patient in the primary tumor and in surrounding lymph nodes. According to our knowledge, no similar study has been published yet.

Keywords Human papillomavirus · HPV · Squamous cell cancer · Colon cancer · PCR · Southern blot hybridization

Introduction

Human papillomaviruses (HPV) have icosahedral DNA spiral, and are papomaviruses encapsulated with a protein envelope [1]. More than 70 subtypes were identified and their role is well known in the pathogenesis of a number of benign and malignant diseases. In benign diseases the most frequently identified subtypes are HPV 6 and 11, while in malignant tumors HPV 16 and 18 were found most frequently. It has been proved that three HPV genes, E5, E6 and E7 are responsible for changes induced in the proliferation of the host cell [2, 3]. E5 gene is encodes a protein that activates epidermal growth factor. E6 and E7 express their oncogenic activity by the inhibition of p53 and Rb suppressor genes. The role of HPV in malignant transformation has been proved in several organs, e.g. in cervical and anal carcinomas, malignant tumors of the head–neck region as well as in esophageal cancer [4] and colorectal cancers (CRC) within the gastrointestinal system [5–7]. Squamous cell carcinoma (SCC) is a rare form of CRC. Because of its squamous cell origin, the possible pathogenetic role of HPV can be raised. Since this disease is very rare, only case reports of HPV infection have been published yet [8, 9]. Even in the study investigating the largest patient population there were only six cases of HPV infection in real SCC patients involved [10]. Until now no HPV test results have been published on lymph nodes surrounding colorectal tumors. The objective of our work was to detect the presence of HPV not only in the primary tumor but also in the surrounding removed lymph nodes.

Material and Methods

Patient and Material

The 94-year-old female patient was hospitalized 4weeks earlier due to passage disturbances. The performed tests (pelvic X-ray, abdominal USG, colonoscopy, oesophago-gastroscopy, and later CT) confirmed a significant space reducing tumour in the sigmoid colon. During exploration

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we found a tumor in the upper third of the sigmoid colon. Neither liver metastasis nor peritoneal dissemination or ascites were observed. Segmental resection of sigmoid colon was performed. No surgical or postoperative complications were observed. The patient was discharged 8days after surgery. The histologic diagnosis was: non-keratoid planocellular (squamous) carcinoma with anaplastic components (grade 3) with metastases the regional lymph nodes (2:9); staging: Dukes C1, T2, N1, Mx. To exclude metastatic origin of the tumor (Fig. 1.) CT was performed and proved negative.

DNA Extraction

Five sections (5μm) were cut from each paraffin block and placed in a 500-μl Eppendorf tube. One section was stained with hematoxylin and eosin for histological observation. DNA was extracted with DNA Mini Kit (Qiagen, USA) using modified protocol with digestion with 180μl ALT Buffer and 30μl Proteinase K for 16h at 56°C. All procedures were performed carefully to avoid contamination.

Polymerase Chain Reaction (PCR)

For detection of HPV DNA, broad-spectrum consensus and highly specific (-16, -18) primers and probes were used (Table 1). PCR was performed essentially as described earlier. [11] Briefly, the "master mix" contained 78μl distilled water, 6μl dNTP nucleotide mix (Fa. Boehringer, Mannheim, Germany), 3–3μl primer-1 and primer-2, 30μl PCR incubating buffer, 60μl Solution-Q and 36μl $MgCl_2$ (25mM). To 18μl of this mixture 2μl of eluated DNA was added. Samples were then denatured for 10 minutes at 95°C. After adding of 5μl Taq-polymerase solution (PCR kit, Qiagen, Foster City, USA), 40 cycles were performed using beta-globine and consensus primers, consisting of annealing for 60s at 55°C, extension for 60s at 72°C and denaturation for 60s at 94°C. For HPV-16 and HPV-18 primers 45 cycles were performed with annealing for 40s at 57°C, extension for 40s at 72°C and denaturation for 40s at 94°C. As controls we used beta-globine and standard HPV-DNA (Prof. de Villier, ZfKF, Heidelberg). Amplified DNA (14μl) was electrophoresed with 2μl bromophenolblue on 2% TEA-agarose gel, using 80V for 2h, and made visible by ultraviolet light after staining with ethidium bromide.

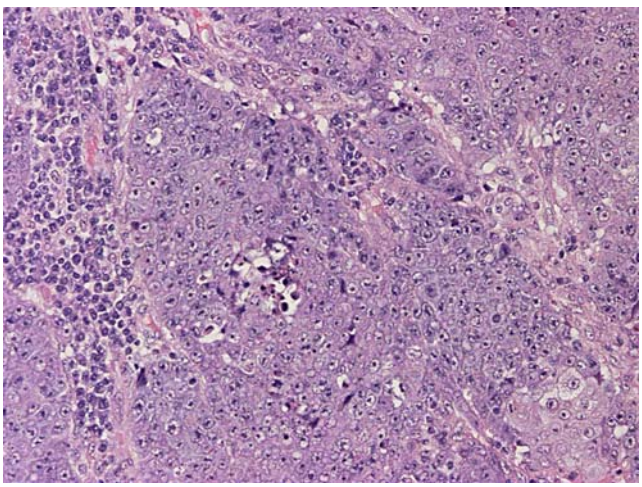

Fig. 1 Squamous cell cancer of sigmoid colon (HE)

Table 1 Primers and probes used for detection of human papilloma virus infection in squamous cell cancer of colon and regional lymph nodes

Primers	Nucleotide sequences
HPV-consensus	GP 5.1: 5′ TTTGTTACTGTGGTAGATAC 3′
	GP 5.2: 5′ GAAAAATAAACTGTAAATCA 3′
	Product size, 139–154 bp
HPV-16	5′ TCAAAAGCCACTGTGTCCTG 3′
	5′ CGTGTTCTTGATGATCTGCA 3′
	Product size, 120 bp
HPV-18	5′ CAGTATACCCCATGCTGCATGCC 3 ′
	5′ CGGTTTCTGGCACCGCAGGCACC 3′
	Product size, 160 bp
Betaglobine	PC 03: 5′ ACACAACTGTGTTCACTAGC 3′
	PC 04: 5′ CAACTTCATCCACGTTCACC 3′
	Product size, 110 bp
Probes	Sequences
HPV-16	5′ CATAATATAAGGGGTCGGTGGACCGGT 3′
HPV-18	5′ CAGACTCTGTGTATGGAGACAC 3′
Betaglobine	19 A: 5′ CTCCTGAGGAGAAGTCTGC 3′

Fa. B & G Biotech GmbH, Freiburg, Germany

Southern Blot Hybridization

After staining with ethidium bromide, the DNA was transferred to nitrocellulose by Southern's method [12]. The filters were hybridized with [32] P-5′ end labeled oligonucleotide probes under stringency conditions using the Oligonucleotide Tailing Kit (Boehringer, Germany). After hybridization with 60μl oligonucleotide for 16 hours at 56°C, the filters were washed under low stringency condition at room temperature. Anti-digoxigenin-AP-conjugate was used for visualization (DIG-Nucleotide Acid Detection Kit, Boehringer).

Results

We could identify beta-globin from DNA obtained from the examined SCC and lymph nodes in every case, confirm successful DNA extraction. Results obtained from the PCR tests performed with consensus primers were similar to

those obtained with the HPV-16 primers. Presence of HPV-18 could not be confirmed either in the tumor or the lymph nodes. We found HPV-16 infection in four lymph nodes. Out of the nine tested lymph nodes two were metastatic; both were infected with HPV-16. HPV-16 infection was confirmed in two of the seven negative lymph nodes as well, and in one case of these only Southern blot hybridization could proved the presence of the virus (Fig. 2).

Discussion

A total of less than 150 cases have been reported [14] since Martha Schmidtmann described colorectal SCC in 1919 [13]. However, the incidence of the disease is presumably higher. Audeau has found 20 cases in 2,351 CRC patients (0.85%), out of which the number of real SCC was six; the other patients had adeno-squamous carcinoma and adeno-carcinoma with squamous metaplasia [10]. It is not clear whether or not these can be considered as a separate entity [14], however, the fact is that all SCC in the above cases were found in the rectum. SCC located in the colon is surely much less frequent. Determination of primary colorectal SCC has strict criteria [15]: (a) No squamous carcinoma with other location can be diagnosed to exclude direct metastasis. (b) There cannot be epithelial fistula in the relevant colon segment. (c) The tumor may not reach the level of anorectal junction. (d) The tumor may not show glandular differentiation. Our case met these criteria. A number of causes were considered as the etiology of primary colorectal SCC: (a) Proliferation within persisting ectopic embryonic ectodermal cells, transformation to squamous cell, and malignant alteration [16]. (b) Development from on the base of on chronic inflammations, e.g. ulcerative colitis [17–19] from undifferentiated cells due to irritation or injuries of the mucous membrane. (c) From chronic infections, e.g. schistosomiasis [20, 21] or HPV infection. (d) Development on squamous metaplastic islands within colorectal adenomas [22]. Finally (e) it was also arisen that colorectal SCC is an undifferentiated cancer with squamous components, thus it is not a separate entity [14]. The etiological role of HPV in anal cancer is well known. One case of HPV infection in colorectal SCC has been reported [9]. Only two studies investigated SCC; in one study 6 cases were HPV-negative [14], while in the other a total of 20 colorectal cancers also showing squamous components were identified, but only in six of these were SCC the pathological diagnosis, and all of them were located in the rectum [10]. The above investigations were performed with the less sensitive in situ hybridization. Although the test is specific, its sensitivity is between 50% and 70% [23]. PCR is the gold standard in HPV detection. In the only HPV-positive colorectal SCC case the virus was identified with PCR, moreover, with the nested RT-PCR the transcription activity of HPV E6/E7 oncogene has been confirmed in the periferial part of the tumor and in the normal mucosa next to the tumor [9]. By using PCR technique HPV infection could be detected in colorectal carcinomas as well. In one case HPV DNA was detected in 11 of 37 adenomas, and in 37 of 70 CRC cases by using PCR and Southern blot hybridization [24]. In another study on 19 CRC cases, HPV-18 infection was confirmed in 16 cases in the tumour an in ten cases in the normal mucosa [25]. Other authors, however, did not find HPV infection in CRC [26, 27]. The reason for such differences may be the well-known geographic distribution of HPV. In esophageal cancer patients the incidence of HPV in the so-called high risk regions (e.g. China, Taiwan, Pakistan, Iran, South Africa, and France) is noticeably higher [4]. Also, the study mentioned above proving the infection was published by Taiwanese and Chinese authors. In our case HPV infection could be confirmed in the metastatic as well as in tumour-free lymph nodes. This corresponds to the study that confirmed HPV transcription activity also in the normal mucosa along the tumor. It was mentioned previously that HPV is responsible only for the initiation of the malignant

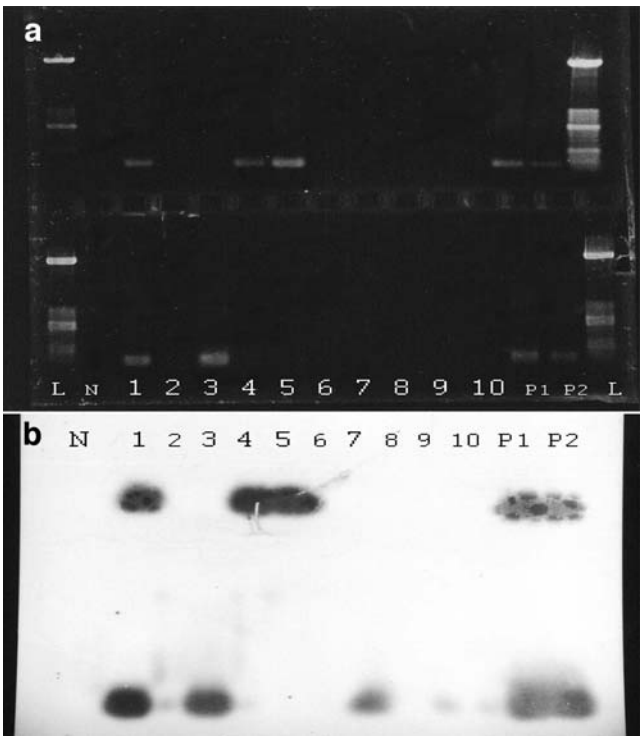

Fig. 2 Detection of HPV-16 by PCR (**a**) and Southern blot hybridization (**b**). *L* 50-bp DNA ladder, *N* negative control, *P1* 1 ng HPV-16 DNA, *P2* 0.5 ng HPV-16 DNA, *lane 1* primary tumor, *lane 2* no sample, *upper lanes 3, 6 and lower lanes 3–7* non-metastatic lymph nodes, *upper lanes 4, 5* metastatic lymph nodes

transformation, and in many cases it cannot be detected from the developed malignant tumor anymore [4]. In the transcription phase the patient does not have either symptoms or detectable tumor, thus the virus cannot be identified. In early esophageal tumors and certain benign lesions HPV infection could be confirmed with a similar ratio compared to the developed malignant tumours according to the distribution characteristic to the geographic region. The virus appearing in the lymph node suggests lymphogen distribution of the pathogen also in this tumor type. This was also confirmed in case of cervical cancers which were extensively studied in terms of HPV infection [28, 29].

In summary, primary colorectal SCC is a rare disease and its etiology is unclear. The etiological role of HPV known in other squamous tumours has not been confirmed in colorectal SCC. In our case we could identify HPV 16 infection in the tumour tissue, in the metastatic lymph nodes and in two negative lymph nodes. Based on literature data it can be concluded that the SCC case described above has been developed on the basis of HPV infection.

References

1. McMurray HR, Hguyen D, Westbrook TF, McAnce DJ (2001) Biology of human papilloma viruses. Int J Exp Pathol 82:15–33
2. Mantovani F, Banks L (2001) The human papillomavirus E6 protein and its contribution to malignant progression. Oncogene 20:7677–7685
3. Nguyen DX, Westbrook TF, McCance DJ (2002) Human papilloma virus type 16 E7 maintains elevated levels of cdc25A tyrosone phosphatase during deregulation of cell cycle arrest. J Virol 76:619–632
4. Syrjanen KJ (2002) HPV infection and oesophageal cancer. J Clin Pathol 55:721–728
5. Perez LO, Abba MC, Laguens RM, Golijov CD (2005) Analysis of adenocarcinoma of the colon and rectum: detection of human papillomavirus (HPV) DNA by polymerase chain reaction. Colorectal Dis 7:492–495
6. Bodaghi S, Yamanegi K, Xiao SY, et al (2005) Colorectal papillomavirus infection in patients with colorectal cancer. Clin Cancer Res 11:2862–2867
7. Buyru N, Budak N, Yazici M, Dalay N (2003) p53 gene mutations are rare in human papillomavirus-associated colon cancer. Oncol Rep 10:2089–2092
8. Michelassi F, Mishlove LA, Stipa F, Block GE (1988) Squamous cell carcinoma of the colon: Experience at University of Chicago, review of the literature, report of two cases. Dis Colon Rectum 31:228–235
9. Sotlar K, Koveker G, Aepinus C, et al (2001) Human papilloma virus type 16 associated primary squamous cell carcinoma of the rectum. Gastroenterology 120:1046–1048
10. Audeau A, Han HW, Johnston MJ, et al (2002) Does human papilloma virus have a role in squamous cell carcinoma of the colon and upper rectum? Eur J Surg Oncol 28:657–660
11. Bognár G, Imdahl A, Ledniczky G, Ondrejka P (2006) A HPV infekció lehetséges prediktív szerepe a nyelőcsőrákos betegek neoadjuváns terápiára adott remissziójában. Magyar Sebészet 59:97–104
12. Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517
13. Schmidtmann M (1919) Zur Kenntnis seltener Krebsformen. Virchows Arch B Pathol Anat 226:100–118
14. Frizelle FA, Hobday KS, Batts KP, Nelson H (2001) Adenosquamous and squamous carcinoma of the colon and upper rectum. A clinical and histopathological study. Dis Colon Rectum 22:341–346
15. Cooper HS (1989) Carcinoma of the colon and rectum. In Norris HT (Ed.) Pathology of the colon, small intestine and anus, p 201. Churchill Livingstone, New York
16. Grivalinsky HT, Helwig EG (1956) Carcinoma of the anorectal junction. Histological considerations. Cancer 9:480–488
17. Hohm WH, Jackman RJ (1964) Squamous cell carcinoma of the rectum complicating ulcerative colitis. Mayo Clin Proc 39:249–251
18. Goldgraber MB, Humphreys EM, Kirsner JB, Palmer WL (1960) Carcinoma and ulcerative colitis: clinicopathologic study. Gastroenterology 34:809–838
19. Michelassi F, Montag AG, Block GE (1988) Adenosquamous cell carcinoma in ulcerative colitis: Report of a case. Dis Colon Rectum 31:323–326
20. Weiner MF, Polayes SH, Yidi R (1962) Squamous carcinoma with schistosomiasis of the colon. Am J Gastroenterol 37:48–54
21. Pittella JE, Torres AV (1982) Primary squamous cell carcinoma of the colon and ascending colon: report of a case and review of the literature. Dis Colon Rectum 25:483–487
22. Williams GT, Blackshaw AJ, Morson BC (1979) Squamous carcinoma of the colorectum and its genesis. J Pathol 129:139–147
23. Yun K, Sherwood MJ (1992) *In situ* hybridisation at light and electron microscopic levels: indentification of human papilloma virus nucleic acids. Pathology 24:91–98
24. Cheng JY, Sheu LF, Meng CL, et al (1995) Detection of human papilloma virus DNA in colorectal carcinoma by polymerase chain reaction. Gut 37:87–90
25. Lee YM, Leu SY, Chiang H, et al (2001) Human papilloma virus type 18 in colorectal cancer. J Microbiol Immunol Infect 34:87–91
26. Shah KV, Daniel RW, Simons JW, Vogelstein B (1992) Investigation of colon cancer for human papillomavirus genomic sequences by polymerase chain reaction. J Clin Oncol 51:5–7
27. Gilbert JM, Mann CV, Scholefield J, Domizio P (1991) The aetilogy and surgery of carcinoma of the anus, rectum and sigmoid colon in Crohn's disease. Negative correlation with human papillomavirus type 16 (HPV 16). Eur J Surg Oncol 17:507–513
28. Likaszuk K, Liss J, Wozniak I, et al (2004) HPV and histopathological status of pelvic lymph node metastases in cervical cancer: a prospective study. J Clin Pathol 57:472–476
29. Füle T, Csapó Z, Máthé M, et al (2006) Prognostic significance of high-risk HPV status in advanced cervical cancers and pelvic lymph nodes. Gynecol Oncol 100:570–578