Journal of BUON **14:** 495-499, 2009

ORIGINAL ARTICLE

Detection of human papillomavirus DNA by polymerase chain reaction and southern blot hybridization in colorectal cancer patients

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Summary

Purpose: *The molecular mechanisms related to colorectal carcinogenesis are controversial. The purpose of this study was to evaluate the possible role of high-risk oncogenic human papillomavirus (HPV) types in the pathogenesis of colorectal cancer.*

Patients and methods: *Tumor, and corresponding normal mucosal tissue specimens were obtained soon after surgery from 56 patients with colorectal adenocarcinoma. We studied both neoplastic and normal colon tissues for the presence of HPV types 6, 11, 16, 18, and 33. After the isolation of DNA, the presence of specific types of HPV DNA was determined by polymerase chain reaction (PCR) and southern blot hybridization.*

Results: *HPV DNA was detected in 46 (82.14 %) of 56 colorectal adenocarcinomas and in 18 (32 %) of 56 normal colonic mucosal tissue samples. Two or more HPV types were detected in 32 carcinoma samples. HPV type 18 (n= 40) and 33 (n= 32) were the most frequently detected types of HPVs in the tumor tissues. None of the normal mucosal specimens revealed HPV 18 DNA. The expression rate of HPV DNA in tumor tissue was significantly higher than that encountered in normal colonic mucosa ($p < 0.001$).*

Conclusion: *Detection of HPV DNA types 18 and 33 in most of the colorectal adenocarcinoma specimens suggests that HPVs may be related to carcinogenesis in glandular cells of the colorectal mucosa of our patient population.*

Key words: carcinoma, colon cancer, human papillomavirus, PCR, rectal cancer, viral association

Introduction

Adenocarcinoma of the colon and rectum is the second most common cause of cancer death in the United States and in most other western countries [1]. Although numerous genetic, dietary and epidemiological factors have been proposed, the precise etiology of colon cancer is not known. HPV is a DNA virus that has been recognized as an etiological factor in many types of cancer such as esophageal cancer [2], lung cancer [3], head and neck cancers [4], breast cancer [5] and cervical cancer [6-9]. Surprisingly, although papillomaviruses were the first virus family found to transmit cancer [10,11], their carcinogenic potential in the development of colorectal cancer has not been studied thoroughly.

In humans, more than 80 types of HPVs have been described. A common feature of these viruses is their epitheliotropism. All types of epithelium including skin and mucosal surfaces may be infected by HPV. Histologically, normal epithelium may contain HPV DNA [12]. HPV infection is transmitted by close personal contact. Anogenital HPV infections are the most common sexually transmitted diseases [13]. HPVs vary in their ability to transform epithelium. In general, there are cutaneous and mucosotropic groups. The cutaneous

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Received 15-11-2008; Accepted 22-12-2008

group (eg. HPV 1,4,5,14,15, 17, 19, 49 and 65) infects keratinized epithelium. The mucosal group (eg. HPV 6, 11, 16, 18 and 33) includes the mucosotropic HPVs that have a higher propensity to be associated with cancer [14-16]. In the mucosotropic group, the viruses may be broadly subdivided into those with low-risk oncogenic potential and those with moderate to high-risk potential. HPV types 6 and 11 primarily cause benign exophytic genital neoplasms [17,18]. The moderate and high risk types include HPV 16, 18, 31, 33, 35, 39, 45, 52, 58, 59 and 67 [16,17]. HPV 18 is most commonly associated with squamous cell carcinoma and adenocarcinoma of the uterine cervix [19].

Accumulating data support the view that HPV may play a causal role in benign and malignant lesions of the colon and rectum [20-25]. In contrast to these studies, there are some reports that have failed to demonstrate the presence of HPV in tissue samples of colorectal cancer, even though they used the same methods of detection [26,27]. Thus, further studies involving large number of patients with colorectal carcinoma are required to explain these contradictory findings.

To investigate the relationship between HPV and colorectal adenocarcinoma, we prospectively examined 56 colon carcinomas and the corresponding normal colonic mucosa samples of the patients for HPV sequences by PCR and southern blot hybridization.

Patients and methods

Tumor tissue and corresponding normal mucosal tissue specimens were obtained from 56 patients with colorectal adenocarcinoma who had undergone surgery in the Department of Surgery at Istanbul University Medical Faculty between December 1995 and November 1996. Signed informed consent was obtained from each patient. The study was approved by Istanbul University School of Medicine Ethics Committee.

Patients

The patients' age ranged from 26 to 74 years. Thirty-two patients were male and 24 female. None of the patients had a previous history of cervical cancer or dysplasia or presented clinical evidence of HPV infection elsewhere in the body. Patients with diagnosis of familial adenomatous polyposis, hereditary non-polyposis colon cancer, inflammatory bowel disease, or immunosuppressive conditions were excluded from the study. Other exclusion criteria were prior total or partial colectomy for benign or malignant colorectal diseases, and radiation and/or cytotoxic therapy for colorectal adenocarcinoma or other malignancies (except adequately treated basal /squamous cell carcinoma or cervical carcinoma *in situ*) within the previous 5 years.

Methods

The diagnosis was done based on the presence of adenocarcinoma in tissues obtained by colonoscopic biopsy and confirmed postoperatively by histopathologic examination of the resected colon segment. The tissue samples of both carcinoma and normal mucosa were freshly obtained soon after the operation by dissecting the resected colon segment and were kept at –20^{o} C when not immediately processed.

After overnight incubation with proteinase K, DNA was isolated by phenol chloroform extraction. The HPV-specific sequences were amplified by PCR. The primers used were: MY11; 5' GCMCAGGGWCATAAYAATGG –3' and MY09; 5' CGTCCMARRGGAWACTGATC-3'. These primers amplify a region about 450 bp from the L1 open reading frame. DNAs from lymphocytes and from an HPV-positive patient were used as negative and positive controls, respectively. The PCR reaction mixture contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.08 % Nonidet P4O , 4 mM $MgCl_2$, 200 mM of each dNTP, 1.25 U of Taq polymerase and 100 ng of patient DNA in 50 µl final volume. Beginning with incubation at 95^{o} C for 5 min, amplification was performed for 35 cycles of denaturation at 95^{o} C for 30 sec, annealing at 55^{o} C for 30 sec and extension at 72^{o} C for 1 min. The reaction was brought to an end by extension at 72^{o} C for 10 min. Aliquots of 9 µl of each product were electrophoresed in 1.5% agarose gels containing 0.5 mg/ml ethidium bromide and the gels were photographed under ultraviolet light.

For southern blot hybridization, 20 µl of the PCR product were transferred from the gel to a nylon membrane (Sure Blot, Oncor Inc, Gaithersburg; MD) by diffusion blotting in 0.5 M NaOH, and 0.6 M NaCl . The membrane was neutralized with 0.1 × SSC (sodium chloride, sodium citrate solution) and the blots were dried at room temperature for 5 min. Prehybridization was performed at 68^{o} C in a solution containing 5 × SSC (0.75 M NaCl, 0.075 M Na-citrate, pH: 7.0), 1% blocking reagent, 0.02% SDS (sodium dodecyl sulfate), 0.1% lauryl sarcosine, and 45% formamide. Hybridization was performed in the same solution at 45^{o} C for 12 h with 3'-DIG- labeled oligonucleotide probes for HPV 6, 11, 16, 18 and 33 types (Boehringer Mannheim, GmbH). The blots were washed 3 times in

0.16 SSC + 0.1 % SDS for 15 min at room temperature and once in 0.16 × SSC + 0.1% SDS for 35 min at 60° C. When hybridization was completed 150 mU/ml of AP-conjugate was added. After binding, the membrane was washed to remove free antibody and incubated with NBT / BCIP (nitro blue tetrazolium /5-bromo-4-chloro-3-indolyl phosphate) in the dark for 12 h for color development.

Groups were compared using x^2 analysis. Statistical significance was accepted at the 5 percent level.

Results

The detection rate of HPV DNA varied dramatically between normal mucosa and tumor tissues. While only 18 (32%) samples of normal colon mucosa contained HPV DNA, 46 (82.14 %) samples of tumor tissue samples displayed HPV genomic sequences ($p < 0.001$). While in 32 carcinoma samples 2 or more HPV types were detected, in 14 tumor tissue samples only one HPV type was observed. HPV 18 was the most frequently detected HPV type in tumor tissue. It was detected in 40 (71.4%) samples of 56 colorectal adenocarcinomas. In contrast, no HPV 18 was detected in the 56 normal colonic tissue specimens. Although, the HPV 16 detection rate was higher in cancer tissue samples than in normal mucosal samples, the difference was not statistically significant (Table 1). Similarly, the distribution of HPV types 6 and 11 in normal mucosa was not significantly different from that in tumor tissue ($p > 0.05$). HPV 33 was the second most common HPV type identified in the cancer samples. The detection rate of HPV types 18 and 33 in tumor tissues was significantly higher than the rate seen in normal mucosa ($p < 0.001$).

Discussion

The majority of colorectal adenocarcinomas are believed to arise from adenomatous polyps. It appears that <1% of polyps ever become malignant. The probability of an adenomatous polyp becoming cancerous is dependent on a multistep process that includes altered proliferative pattern of the colonic mucosa coupled to the loss of mechanisms that normally suppress tumorigenesis (the polyp-cancer sequence).

The E6 and E7 proteins of HPV have the ability to immortalize epithelial cells by interacting with p53 and pRb respectively [28-30]. The interaction of the E6 protein with p53 targets p53 for degradation via an ubiquitin-dependent pathway [31,32], and interaction of E7 with pRb renders the latter protein inactive [33,34]. Thus the E6 and E7 proteins may trigger the events that induce epithelial cells of colonic mucosa to proliferate indefinitely. Moreover, the mechanisms regulating these changes in cell proliferation have been defined by the demonstration that transfection of normal human cervical epithelial cells, epidermal keratinocytes, and laryngeal epithelial cells with either HPV 16 or HPV 18 result in a 5- to 10-fold increase in epidermal growth factor receptor (EGFR) [35,36]. This increased expression is associated with a more aggressive phenotype [37,38]. All of these mechanisms may explain the polyp-cancer sequence of colorectal cancers.

We have demonstrated the presence of HPV genomic sequences in 46 (82.1%) of 56 colorectal carcinomas, and in 18 (32.1%) of 56 normal colonic mucosal specimens. This difference in HPV DNA detection in colorectal cancer and normal colonic mucosa was statistically significant. In previous studies HPV DNA positivity in colorectal cancer ranges from 0% to over 80% [25,27]. In a study of archival specimens, Kirgan et al. identified HPV genomic sequences in 13/38 carcinomas, 8/21 adenomas and 2/24 normal colon mucosa, with PCR and southern blotting [21]. In the Bodaghi et al. study [23], the authors conducted a retrospective controlled study using tumor and tumor adjacent colorectal tissues dissected from patients with colorectal cancer, as well as colorectal tissues from control individuals with no cancer. They found positive HPV DNA in 42% of the tumor tissues of the patients and in 29% of the tumor-adjacent tissues ($p=0.1$), as compared with none of the controls ($p < 0.05$). Perez et al. [24] investigated tissue samples from Argentinean patients by MY/GP nested PCR, detecting HPV in 74% of colorectal carcinomas. Recently, Damin et al. [25] detected HPV DNA in over 83% of the patients with cancer using the GP5+/GP6+ primers in an auto-nested PCR, but in none of the colorectal tissues obtained from controls without cancer. Our findings support the possibility that HPV infection is involved in the development of colorectal cancer. Similar results were

Table 1. Results of HPV DNA detection by PCR and southern blot hybridization

HPV types	*Tumor (+) cases*	*Normal mucosa (+) cases*	*p-value*
6	4	0	> 0.05
11	2	8	> 0.05
16	10	8	> 0.05
18	40	0	< 0.001
33	32	6	< 0.001

obtained in other studies [20-25]. However, the presence of HPV DNA in colorectal cancer tissue remains controversial. Using PCR methodology, Shah et al. and Shroyer et al. could not show HPV DNA in colon biopsies [26,27]. In the first of these studies Shah and colleagues examined 50 colon carcinomas including 19 primary tumors, 15 nude mice xenografts, and 16 established cell lines for HPV sequences by PCR, and failed to detect HPV genomes in any of the tumors [26]. In the second of the negative studies, Shroyer and co-authors [27] demonstrated HPV DNA in all 5 anorectal squamous cell cancers, but did not find it in any of 22 colon adenocarcinoma tissues.

HPV 18 was the most prevalent type in colorectal adenocarcinomas in our study (40 of 46 positive colorectal adenocarcinomas, 86.9%). Similarly, a study in Taiwan also reported a high frequency of HPV 18 infection in their colorectal cancer patients [39]. However, contrary to our results, other studies have shown that HPV 16 infection was the viral type most frequently detected in patients with colorectal cancer [19-21]. The detection rate of HPV 18 ranged from 10 to 84% in the recently published reports [22-25,39]. These discrepant results in the literature concerning the detection of HPV DNA in colorectal cancer and in the frequency of HPV types may be due to methodological differences, such as study design, sample size, sample collection, and different methods used for analysis. Regional variations in the prevalence of HPV infection, ethnic and geographic origin of the patients may also create these discrepancies.

It has been previously demonstrated that cervical adenocarcinoma was primarily associated with HPV 18 [6,19]. Detection of HPV 18 in colorectal adenocarcinomas in our patient population indicates that HPV 18 may be capable of inducing carcinogenesis in glandular cells of both the colorectal mucosa and the cervical epithelium.

There are some weak points of this study. First, although the corresponding normal mucosal tissue specimens of our colorectal cancer patients were used as control group, it would have been worthwhile studying also the rate of HPV DNA in normal colonic specimens taken from patients with benign colorectal disease. The second weak point of the study is the lack of follow up of our patients. If the patients enrolled into the study were followed long enough then the meaning of the presence of HPV DNA as a prognostic factor might have been clarified.

In conclusion, the ubiquity of HPV infection in colorectal adenocarcinoma makes it highly likely that the initiating events in colorectal carcinogenesis may be the direct results of an HPV infection. Our results suggest that HPVs play a causal role in the pathogenesis of colorectal adenocarcinoma. Thus, HPV vaccination applied for cervical cancer prevention may also decrease the incidence of colorectal cancer in female patients in the future. We also would like to note that HPV 18 and 33 are the most frequently involved types of HPV in colorectal carcinogenesis in our patient population.

Acknowledgement

This study was supported by the Research Fund of Istanbul University, Project number: 835/190496.

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