

Detection of human papillomavirus DNA in colorectal carcinomas by polymerase chain reaction

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Abstract

Human papillomaviruses (HPVs) are associated with a number of benign and malignant neoplasms. To substantiate the relationship between HPV DNA and colorectal carcinomas, 70 carcinomas and 37 adenomas were analysed in this study. Specific types of HPV DNA in colorectal tumours were detected by polymerase chain reaction (PCR) and Southern blot hybridisation. HPV DNA was detected in 11 of 37 (29.7%) adenomas and in 52.9% of 70 (52.9%) of carcinomas. The expression of HPV DNA in adenomas and carcinomas, especially that of HPV 16 in HPV positive cases (4 of 11 v 26 of 37), was significantly different ($p < 0.05$). There was no correlation, however, between HPV and the location, differentiation, stage, or survival of malignant neoplasms. These data suggest that HPV DNA, especially type 16, is associated with colorectal carcinogenesis.

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Keywords: human papillomavirus DNA, colorectal cancer, polymerase chain reaction.

Papillomaviruses are responsible for a wide variety of papillomatous proliferations in animals and humans.¹ They seem to interact synergistically with chemical and physical carcinogens, and thus resemble tumour promoters in function.² The finding that specific types of HPVs are frequently associated with specific types of human tumour has been reported in the past few years.³ It has been suggested that condyloma (genital warts) and mild forms of dysplasia usually contain HPV 6 or 11.^{4,5} HPV 16 and 18, however, are consistently associated with severe dysplasia, carcinoma in situ, and invasive carcinoma.⁶⁻⁸ Most external anogenital neoplasias and cervical carcinomas are associated with HPV 16, and more rarely with HPV 18 or HPV 33.^{9,10} Based on these studies, HPV may play an important part in inducing specific types of human cancer. Although HPVs have a close correlation with cervical carcinoma, they have also been found in adenocarcinomas in a few reports.^{11,12} We have detected HPVs in colon cancer cell lines¹³ and have shown that HPV may be implicated in colorectal carcinogenesis.¹⁴ The expression of different HPVs in clinical specimens of colorectal tumours, however, has never been studied fully.

Polymerase chain reaction (PCR) is the only diagnostic method sensitive enough to detect latent HPV infections.¹⁵ One copy of HPV DNA in 10^5 cells¹⁶ or 20 viral copies in cervical cancer biopsy tissue¹⁷ can be detected by PCR. PCR can be used currently to amplify DNA in paraffin embedded sections.¹⁸ We describe the expressions of HPV types 6, 11, 16, 18, and 33, detected by PCR and Southern blot hybridisation and substantiate their possible role in colorectal tumours.

Methods

CLINICAL SPECIMENS AND CELL LINES

Paraffin embedded specimens of 37 adenomas and 70 carcinomas were collected for this study. All clinicopathological data including sex, age, histological differentiation, tumour stage, tumour location, and survival years were recorded. The five year survival status of all patients was also collected. The cases of colorectal cancers were classified by Astler and Coller's classification¹⁹: eight cases were stage A, 24 stage B (B1+B2), 35 stage C (C1+C2), and 11 cases stage D. A cell line containing HPV 16, CaSki, and another containing HPV 18, HeLa, were used as positive controls. The DNA of 10 normal colonic epithelia was used as a negative control.

DNA EXTRACTION

Five sections (5 μ m) were cut from each paraffin block and placed in a 500 μ l Eppendorf tube. One section was stained with haematoxylin and eosin for histological observation. The sections were deparaffinised by adding 200 μ l of xylene, they were vortexed, and were then pelleted by centrifugation. Xylene was decanted, and its residue was removed with 200 μ l of 99.5% ethanol. The specimens were then centrifuged and decanted again. After desiccation, 10 mM Tris buffer solution, pH 7.5, was added to each sample. The sections were digested at 37°C overnight with proteinase K (Sigma, St Louis, MI, USA) at 100 μ g/ml in extraction buffer (10^{-2} M Tris-HCl, pH 7.5, 1 mM EDTA, pH 7.9, sodium dodecyl sulphate (SDS) 0.5%). The tube was then heated at 100°C for 10 minutes to inactivate the proteinase K. All the procedures were performed carefully to avoid contamination.

PCR

The E6-E7 region of the HPV genome was

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TABLE I Primers and probes for DNA amplification of human papillomavirus (HPV) polymerase chain reaction

HPV	Nucleotide sequences	Product size (bp)
Primers:		
HPV 6/11	5'-TACTACTGCTGGACAACATGC-3' 3'-CACACAGGGTAGACGCGTG-5'	301
HPV 16	5'-CCCAGCTGTAATCATGCATGGAGA-3' 3'-ACCTTCTGGACAATTACCCGTGTG-5'	253
HPV 18	5'-CGACAGGAACGACTCCAACGA-3' 3'-TCAATTTAGTAGTTGTAATGGTTCG-5'	201
HPV 33	5'-GCAGTAAGGTACTGCACGACTATG-3' 3'-TGCTAAAGTATTATAAAGCCCAGC-5'	413
Probes:		
HPV 6	5'-CAGTGTACAGAAACA-3'	
HPV 11	5'-CAACTACAAGACCTTTTGC-3'	
HPV 16	5'-CCGGACAGAGCCATTAC-3'	
HPV 18	5'-TAAGGCAACATTGCAAGACA-3'	
HPV 33	5'-CACAAACATTGAACTACAGTGCCTGGAATGCAAAAAACCTT-3'	

chosen to prepare the primers.²⁰ The sequences of the primers and probes and the amplified length of the HPV viral genome are listed in Table I.

PCR was performed with the Gene Ampli Taq kit (Perkin Elmer-Cetus, Emeryville, CA, USA) by the modified method of Kogan *et al.*²¹ Samples were then denatured for 10 minutes at 94°C and subjected to 35 cycles of amplification. A cycle represents primer annealing for 2 minutes at 55°C, primer extension for 1.5 minutes at 72°C, and denaturation for 1 minute at 94°C. Amplified DNA (10 µl) was electrophoresed on 3% NuSieve agarose gel (FMC Corp, Rockland, ME, USA) and made visible by ultraviolet light after staining with ethidium bromide.

SOUTHERN BLOT HYBRIDISATION

After staining with ethidium bromide, the DNA was denatured in situ with NaOH and transferred to nitrocellulose filter paper by Southern's method.²² The filters were hybridised with ³²P-5' end-labelled oligonucleotide probes under high stringency conditions, as in previous reports.^{13,14} After hybridisation, the filter was washed under low stringency condition at room temperature and finally exposed to an x ray film (Eastman Kodak, Rochester, NY, USA) by an autoradiographic method at -70°C.

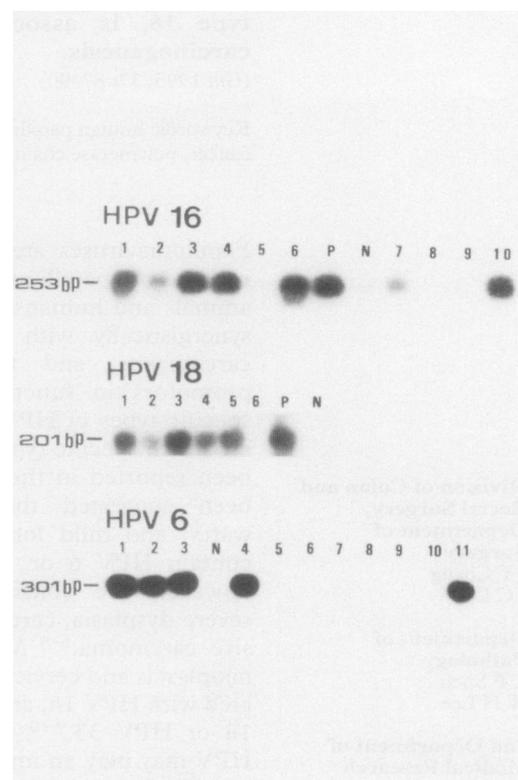
STATISTICS

The difference in cancer differentiation, staging, and location between HPV positive and negative cases were analysed by one-way

analysis of variance (ANOVA) test. The differences in HPVs between adenomas and carcinomas were analysed by the Student's *t* test. This test was also used to analyse the differences between HPVs in respect of patient's sex, age, and five year survival rate.

Results

About 30% (11 of 37) of the DNA extracted from adenomas contained DNA sequences related to HPV 6, 11, 16, or 18. HPVs DNA were found in 52.9% (37 of 70) of 70 carcinomas. The overall incidence of infection by specific HPV types is given in Table II. The difference in HPV infection between adenomas and carcinomas was significant ($p < 0.05$). HPV 16 was the most common HPV found to infect colorectal carcinomas. In addition, there was a significant difference in HPV 16 expression in HPV positive cases of adenoma and carcinoma (4 of 11 *v* 26 of 37) ($p < 0.05$). In the control group, there was no normal mucosa that contained HPV DNA. The Southern blot analysis of HPV infection in colorectal carcinoma is shown in Figure 1. Double infections were found in one of stage B1, one of stage C1, three stage C2, and one of stage D carcinomas respectively. Thus, the numbers of specimens positive for each specific HPV type were total more than



Genomic DNA of colorectal cancers detected by polymerase chain reaction with human papillomavirus (HPV) primer and Southern blot hybridisation. Upper section HPV 16: lanes 1-4, 6, 7, and 10 were positive and lanes 5, 8, and 9 were negative. P=CaSki cell line (positive control) and N=negative control. Middle section HPV 18: lanes 1-5 were positive and lane 6 was negative. P=HeLa cell line (positive control). Lower section HPV 6: lanes 1-4 and 11 were positive and lanes 5-10 were negative.

TABLE II Typing of human papillomavirus (HPV) DNA in the different stages of carcinomas as determined by polymerase chain reaction and Southern blot hybridisation

	HPV				Positive (%)
	6/11	16	18	33	
Normal mucosa*	0	0	0	0	0/10 (0)
Carcinoma:					
Stage A (n=8)	0	2	2	0	4/8 (50)
B1 (n=8)	1	2	1	0	3/8 (37.5)
B2 (n=16)	2	5	2	0	9/16 (56.3)
C1 (n=9)	1	3	1	0	4/9 (44.4)
C2 (n=18)	1	9	3	0	10/18 (55.6)
D (n=22)	1	5	2	0	7/11 (63.6)
Total	6	26	11	0	37/70 (52.9)†

*Control group. †The ratio of HPV positive cases in carcinomas.

TABLE III Incidence of human papillomaviruses (HPV) in a differentiated series of carcinomas

HPV type	Differentiation			
	Well	Moderate	Poor	Mucinous
6/11	1	4	1	
16	2	17	5	2
18	1	8	2	
Total	4/10 (40%)	25/45 (55.6%)	6/12 (50%)	2/3 (66.7%)

HPV type were total more than the number of positive cases in Table II.

There was no significant association between HPV positivity and the cell differentiation of carcinomas ($p > 0.05$) as is shown in Table III. Double infections were found in four cases of moderate and two cases of poor differentiation. The five year survival rate was 37% (10 of 27) in HPV positive cases and 42.4% (14 of 19) in negative cases. There was no significant association between HPV and survival in patients with colorectal cancer. In addition, there was no distinct difference in the mean age, sex or tumour location between HPV positive and negative cases.

Discussion

Although HPV DNA certainly prevails in the genital tract,⁶⁻⁸ it may also be a risk factor for neoplasm in other locations such as the lung,²³ oral cavity,²⁴ larynx,²⁵ skin,²⁶ and tongue.⁵ The association between HPV and colorectal neoplasia has been described in a few studies.¹²⁻²¹ In our previous studies, we found HPV 16 and HPV 18 DNA sequences in the DNA of colorectal cancer cells.¹³ Furthermore, HPV 16 DNA in colon cells could be transfected during the process of malignant transformation.¹⁴ Since Yasumoto²⁷ proved that HPV 16 DNA was closely associated with malignant transformation, HPV 16 DNA has been reported to activate proto-oncogene and play an important part in carcinogenesis.²⁸ These results suggest that HPV might play a causal part in colorectal carcinogenesis.

Based on HPV prevalence rates in cervical carcinomas and in vitro transforming capabilities, HPV 16 and 18 genotypes have been grouped as high risk, and HPV 6 and 11 as low risk for the development of cervical cancers.¹² HPV 6 and 11 have been detected almost exclusively in benign lesions such as laryngeal papillomas, benign condyloma acuminata and low grade cervical intraepithelial neoplasia (CIN).^{1,2}

With regard to the occurrence of specific HPV types in cervical tumours, HPV 16 was the most prevalent in invasive carcinomas.^{6,7} HPV 16 DNA is therefore the predominant genotype to induce cancer. This DNA may integrate into the host genome near the *c-myc* oncogene^{28,29} and activate *c-myc* expression.¹³ In our study, 70% (26 of 37) of HPV DNA positive cases contained HPV 16 DNA. This result suggested that HPV 16 may also have a role in the several stages of colorectal carcinogenesis.

The expressions of HPV DNA in adenomas

and carcinomas were significantly different in our study ($p < 0.05$). In addition, we have also found increased association of HPV with progressive dysplasia in adenomas (unpublished data). Our results were consistent with clinicopathological evidence in previous reports³⁰⁻³² and suggested that most colorectal carcinomas arise from pre-existing adenomas (adenoma-carcinoma sequence). However, there was no correlation between the existence of HPV and differentiation of carcinomas ($p > 0.05$). This finding indicated an important biological difference between adenomas and carcinomas.

We also analysed the associations between HPV and sex, age, tumour stage, tumour location, and survival status. There was no close correlation with any of these factors. The results suggested that viral infections were not retrograde transmission from anus to caecum. Furthermore, HPV DNA may play a crucial part in promoting colorectal carcinogenesis, but not in cancer invasion and metastasis.

We^{13,14} and Kirgan *et al*³³⁻³⁶ have shown HPV DNA in colonic tumours, whereas Palmer *et al*³⁷ and Shroyer *et al*³⁸ did not. This discrepancy may result from different techniques or samples. In the study of Shroyer *et al* for example almost half of the metastatic samples were used. Although the association between HPV and colonic tumours is not confirmed by everyone and more cases will be needed to substantiate it fully, our data suggest that HPV DNA is associated with the development of colorectal carcinoma.

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