Human Papillomavirus 16 E7 DNA and E7 Protein Expression in Chinese Colorectal Carcinoma Patients

Jian Cheng Zhang [1], Gang Wu [1], PeiChun Sun [1], Yi Kang [1], HongGuang Li [1], Bao Ping Zhai[1], Yuan Zeng Zhu[1], Yi Ding[2]

[1]Department of General Surgery, Henan Province Hospital, Zhengzhou, Henan 450003, China
[2]Department of Pathophysiology, Weifang Medical University, Weifang, Shandong 261053, China

Abstract Background The relationship between Human papillomavirus (HPV) 16 infection and the natural course of colorectal adenocarcinoma has not been fully defined. In this study, to investigate the correlation between HPV 16 infection and colorectal carcinoma HPV 16 E7 DNA and E7 protein were observed in 106 patients with primary colorectal adenocarcinoma. **Material and methods** 106 patients with primary colorectal adenocarcinoma were enrolled in this study. Fresh tissues were taken from both the tumors and the adjacent normal area of each patient. HPV16 E7 DNA and E7 protein were detected using polymerase chain reaction (PCR) and immunohistochemistry (IHC). **Results** HPV16 E7 expression was significantly higher in colorectal carcinoma (48/106) than that in adjacent normal mucosa (7/106)（P<0.001）. A correlation was found between HPV16 E7 mRNA expression and tumor locations，4/17 in the ascending colon carcinoma and 25/42 in the rectal carcinoma (P<0.05). Higher HPV16 E7 mRNA expression was also associated with lower Dukes stages（Dukes stages between A and C, P<0.05）. IHC shows HPV16 E7 oncoprotein expressed in the nucleus of both tumor and normal mucosal cells. There was a correlation between the expression of E7 oncoprotein and E7 gene. **Conclusions** Our findings indicated that there was a correlation between colorectal adenocarcinoma and HPV 16 infection. HPV16 infection was relatively higher in the colorectal carcinoma and rare in the adjacent normal mucosa. Specimens expressing higher levels of HPV 16 E7 DNA were associated with lower Dukes stages and more distal locations.

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Key words: colorectal neoplasms; human papillomavirus (HPV); E7 oncogene

Introduction

The correlation between human papillomavirus (HPV) infection and the occurrence and development of tumors has been a focus of study in recent years. Previous studies showed that HPV, especially the HPV 16 viral genome and antigen expressed in the colorectal tumors (Komlos, Kocjan et al.; Kirgan, Manalo et al. 1990; Lee, Leu et al. 2001; Audeau, Han et al. 2002); (Burnett-Hartman, Newcomb et al.) But the results are controversial in different studies. These following may be the causes: different methods of detections; different primers or probes; different sources or numbers of specimens. To clarify the relationship between colorectal adenocarcinoma and HPV 16 infection, a prospective case control study was performed with a magnitude of samples. In this study, 106 patients with primary colorectal adenocarcinoma were collected. Fresh tissues were excised from both the tumors and the adjacent normal mucosa (10 cm away from the tumor border) of the same patient. The expression of HPV16 E7 DNA and E7 oncoprotein were detected using PCR and IHC.

Materials and methods

Patients and Samples: One hundred and six patients with primary colorectal adenocarcinoma, age range 26-81yrs, medium age 56yrs, male/female, 62/44, were collected at the Department of General Surgery, West China Hospital. All the patients had not been treated with chemotherapy, radiation therapy and immunotherapy before operation. Samples were excised from both the tumors and the adjacent normal mucosa (10 cm away from the tumor border) in each patient. Total 212 clinical samples were collected during primary surgery. Immediately following the resection, both cancer tissues (2 samples of each patient) and normal mucosa tissues (2 samples of each patient) were rinsed with sterile 0.9% NaCl solution. Preserve one sample at -70°C for HPV DNA analysis. The other, used for IHC and H&E stained, was formaldehyde-fixed and paraffin-embedded. Tissue sections from all the patients were reviewed by one pathologist, and the diagnoses of all the 212 specimens were reconfirmed histologically.

DNA extraction

DNA was extracted from tissue specimens by a routine procedure of proteinase K digestion and a standard phenol-chloroform-isoamyl alcohol extraction technique (Pattillo, Hussa et al. 1977). DNA was quantified and qualified using ultraviolet spectrophotometry and electrophoresis.

Primers and PCR Protocol

The HPV-16 E7 specific primers were used in our study. The primers were designed after a thorough search of the HPV 16 sequence database (Los Alamos National Laboratory). HPV16 E7 primers: forward primer 5'-CAC GTA GAG AAA CCC AGC TGT AA-3'; reverse primer 5'-GCA GGA TCA GCC ATG GTA GAT T 3' (manufactured by Shanghai Sangon Bioengineering Co.). These primers yielded 297-bp fragment for the HPV 16 E7 sequence. It is the equivalent of 562-858th in the HPV 16 primitive sequence.

PCR amplifications were performed exactly as described previously (Ding, Zhang et al. 2005). Reaction mixes (30µl) contained 0.5 µl of 5 µM forward and reverse primers, 1 µl of template nucleic acid, and 1 U of Taq DNA polymerase (Sigma-Aldrich Co Ltd, Poole, Dorset, UK). 35 cycles of PCR were performed (95°C for 5 min, 94°C denaturation 30 sec, 55°C annealing 30 sec, and 72°C elongation 1 min). The last cycle was followed by a final extension step of 7 min at 72°C. All reactions were performed on a MJ PTC-100 thermal cycler. PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. The UV-illuminated gels were photographed with Polaroid negatives (type 665). DNA from the Caski cell lines was used as a positive PCR control to assess the success of the amplification. PCR reagents without DNA template (no sample added) served as a negative control.

Immunohistochemistry (IHC)

Immunohistochemistry was performed on 4 µm sections with the streptavidin–biotin–peroxidase technique, as reported previously [1]. Antigen retrieval was performed with heat-induced epitope retrieval with 10 mM citrate buffer (pH 6.1). Tissue sections were incubated with a mouse monoclonal antibody against HPV16 E7 oncoprotein (Sc-6981, Santa cruZ, USA) at a 1:100 dilution. The HPV16 E7 antibody was visualized with the avidin-biotin-peroxidase technique (DAKO LSAB kit; DAKO Cytomation, Carpinteria, USA), Stained with 0.02% 3, 3' diaminobenzidine tetrahydrochloride. We examined HPV16-containing cervical carcinoma specimens as a positive control, and used phosphate buffered saline instead of primary antibody as the negative control. Brown staining of the nucleus and perinucleus indicated positivity for HPV 16 E7 oncoprotein. If the brown stained nuclei account is more than 10% on each of the 5 amplification fields（100×）, the sample is regarded as HPV 16 positive.

Statistical analysis

All data were analyzed using Statistical Package software (SPSS 11.0). Chi-square analysis was conducted. If $p<0.05$, it is a statistical significance.

Results

HPV16 DNA expression and the correlation analysis

The DNA was extracted from the sample of patients and amplified by PCR. The PCR products were detected using electrophoresis (Fig.1). HPV16 E7 fragments were shown a clear band of about 300 bp.

HPV16 E7 expression was significantly higher in colorectal carcinoma, 45% (48/106) than that in adjacent normal mucosa, 7% (7/106), ($p<0.001$) (Table 1). Among them, 4 cases of rectal cancer and 3 cases of sigmoid colon cancer were HPV16 positive in both the tumors and the adjacent normal mucosa. A correlation was found between HPV16 E7 expressions and tumor locations，24% in the ascending colon carcinoma and 60% in the rectal carcinoma ($p<0.05$). Gradually increasing tendency from the cancer site of caecum to anal manifested itself (Table 2). Higher HPV16 E7 expression was also associated with lower Dukes stages (Dukes stages between A and C, $p<0.05$) (Table 3). The differentiation degrees of colorectal carcinoma had no relation to the positive rate of HPV16. There were two well-differentiated adenocarcinomas in our experiment, which were negative of HPV16 E7. Expressions of HPV16 E7 in moderate and poorly differentiated colorectal carcinoma were 45%（34/76）and 50%（14/28）. There was no significant difference.

Table 1. Expression of HPV 16 in colorectal adenocarcinoma and normal mucosa

Samples	Positive(cases)	Negative(cases)	Positive Rate (%)
Adenocarcinoma	**48**	**58**	**45**
Normal mucosa	**7**	**99**	**7****

**$p<0.001$, vs. colorectal adenocarcinoma.

Table 2. HPV prevalence according to anatomic location of tumors

Anatomic Location	Positive(cases)	Negative(cases)	Positive Rate (%)
Ascending	**4**	**13**	**24**
Descending/transverse	**6**	**14**	**30**
Sigmoid	**13**	**14**	**48**
Rectum	**25**	**17**	**60***

* p =0.012, the rectal carcinoma vs. the ascending colon carcinoma.

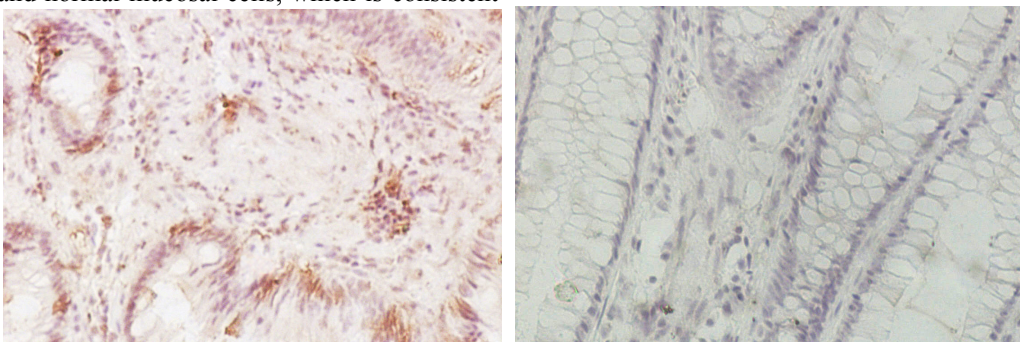
Table 3. HPV 16 positivity in colorectal carcinoma

Dukes stage	Positive(cases)	Negative(cases)	Positive Rate (%)
A	**5**	**15**	**25**
B	**17**	**22**	**44**
C	**16**	**13**	**55***
D	**10**	**8**	**56**

* P =0.036, Dukes stages A vs. C.

HPV16 E7 oncoprotein expression

HPV16 E7 oncoprotein were detected using IHC. HPV16 E7 oncoprotein were expressed in tumor and normal mucosal cells, which is consistent with DNA expression (Fig.2). HPV16 E7 oncoprotein were shown in the nuclear and peri-nuclear of cells.



A (100×) **B** (100×)

Fig.2. The expression of HPV16 E7 oncoprotein

HPV16 E7 oncoprotein were detected using immunohistochemistry. HPV16 E7 oncoprotein were expressed in the nuclear and perinuclear of both tumor and normal mucosal cells (dense brown precipitates). A. Rectal adenocarcinoma, HPV16 E7 oncoprotein positive (100×). B. Colon normal mucosa, HPV16 E7 oncoprotein negative.

Discussion

HPV16 genome is mainly composed of three compartments: early transcribing region (E region), late transcribing region (L region), and upriver regulating region (URR). E6 gene and E7 gene may integrate into the host cellular genome and induce the host cells to be transformed. So it belongs to virus oncogene (Kessis, Connolly et al. 1996). Experimental studies indicated that HPVs, especially HPV16, were one of the etiologies of human cervix and anal squamous cell carcinomas (Frisch, Fenger et al. 1999; Bosch and Munoz 2002). Although various methods were used by different researchers to study the relationship between HPVs and the development of colorectal carcinoma, there are still many secrets to be discovered.

Several technique including IHC, in situ DNA hybridization (ISH) and PCR have been used in recent years to study the relationship between HPVs and colorectal carcinoma. Results indicated that HPV correlated genes and proteins could be found in the colorectal adenocarcinoma tissues (Kirgan, Manalo et al. 1990; Cheng, Meng et al. 1993; Frisch, Glimelius et al. 1997; Lee, Leu et al. 2001; Audeau, Han et al. 2002). Kirgan detected HPVs antigens (97%) and HPVs DNA (43%) in colorectal adenocarcinoma tissues by IHC and ISH techniques. He thought HPV16 is one of the etiologies of colorectal carcinoma (Kirgan, Manalo et al. 1990). Audeau did not find HPV16 expression in 20 paraffin fixed specimens of colorectal cancer using IHC technique and believed that IHC has a high specificity but low sensitivity (Audeau, Han et al. 2002). PCR is used to detect the HPV DNA. Shah KV examined HPV sequences of DNA in 50 colorectal tumors by PCR, using HPV consensus L1 primers for

amplification and found all tumors DNA were negative for HPV sequences (Shah, Daniel et al. 1992). Positive rates of HPV infection in the researches are far from consistence. This may be caused by the methods of detection, the source and amount of specimens. PCR technique has the higher sensitivity than IHC and ISH techniques. Different primers or probes were used. HPV16 DNA usually integrates with the genome of cancer cells. After integrating, only E6 and E7 genes are reserved while other genes are mostly lost or strayed. If sequence other than E6 or E7 are chosen as primers, HPV16 is hard to be detected and the positive rates will be lower (An and Fleming 1991). Additionally, fixed specimens wrapped in paraffin results in destroying the integrity of the DNA and protein and thus a negative result comes out (Steinau, Patel et al.). We employed a magnitude of specimens for prospective control study. 106 patients with primary colorectal adenocarcinoma were enrolled in this study. Samples were excised from both the tumors and the adjacent normal mucosa (10cm away from the tumor border) of the same patient. PCR and IHC were applied to detect the expression of HPV16 E7 DNA and viral protein.

HPV16 infection in colorectal carcinoma and the adjacent normal mucosa

McGregor found that HPV existed in colonal adenocarcinoma by using IHC, PCR and Southern blotting. The positive rate was 32% (13/38), while only 2 positive in 24 normal mucosal tissues (McGregor, Byrne et al. 1993). Frisch found the HPVs DNA expression was 86% in 386 anal cancer, but no expression in 20 rectal carcinoma (Frisch, Glimelius et al. 1997; Frisch, Fenger et al. 1999). Cheng, using PCR and Southern blot hybridization technique, found that HPV positive rate was 29.7% (11/37) in colorectal adenoma and 52.9% (37/70) in colorectal carcinoma, whereas all normal mucosa were negative (Cheng, Meng et al. 1993).

HPV16 E7 is the more potent oncogene in anal cancer caused by HPVs. (Thomas, Pitot et al.) We employed fresh samples (colorectal adenocarcinoma and adjacent normal mucosal tissues) collection during operations and selected E7 gene as primer in our study. Our results indicated that the positive rate of HPV16 E7 in colorectal cancer tissues was 45%, and that of normal mucosal tissues of adjacent carcinoma was only 7%. These suggest that HPV 16 infection may be the etiology of colorectal adenocarcinoma. To further understand the role of HPV 16 DNA in colorectal carcinogenesis, Cheng transformed HPV16 DNA from colonic cancer cells into NIH3T3 mouse cells and suggested that HPV16 might be associated with the malignant transformation of colonic cells (Cheng, Meng et al. 1993).

The correlation between HPV infection and the locations of colorectal carcinoma

Cheng found that there was no relation between HPV infection and the locations of colorectal carcinoma by PCR (Cheng, Sheu et al. 1995). While Weinberger's study indicated that the closer to anus the higher rate of HPV infection. The difference was significant (Weinberger, Yu et al. 2004). We found that the positive rate of HPV16 E7 was much higher in the group of rectal cancer than that in the group of ascending colon cancer (60% to 24%). Gradually increasing tendency of the cancer sites from caecum to anal could be seen. We believed that the reason for this distribution was related to sex, which induced retrograde infection from the perineum to the lower gastrointestinal tract. Thus, chances of contamination of HPVs in the sites of rectum and sigmoid flexure were much more than that in transverse or ascending colon.

The correlation between Dukes stage /cell differentiation and HPV infection

Few reports about the relationship between Dukes stages /cell differentiation of colorectal cancer and HPV infection are found. Cheng believed that there is no correlation between those factors (Cheng, Sheu et al. 1995), while Weinberge found that the lower Dukes stages were always associated with the higher rate of HPV infection (Weinberger, Yu et al. 2004). Our results showed that the differentiation degrees of colorectal carcinoma had no relation to the positive rate of HPV16. As to Dukes stages, significant difference can be seen between stage-A and stage-C ($p<0.05$). Higher HPV16 E7 expression was associated with lower Dukes stages.

Conclusions

HPV16 infection exists in about half of the cases of colorectal adenocarcinoma, and is associated with lower Dukes stages and more distal locations. Yet is it the outcome of HPV DNA integrating into host gene after virus infection, which results in the occurrence and development of colorectal cancer? Or is it an "accompanying infection" induced by HPV invading the damaged site of colorectal mucosal which was the outcome of the tumor tissue death and shedding during its development? Do HPVs accelerate the growth of the tumor after the tumor tissue is infected with HPVs? Further studies are needed to answer these questions.

Corresponding Author:
Yi Ding
E-Mail: dingyi6767@163.com
Tel: 86-15153610766; Fax: 86-536-8567177

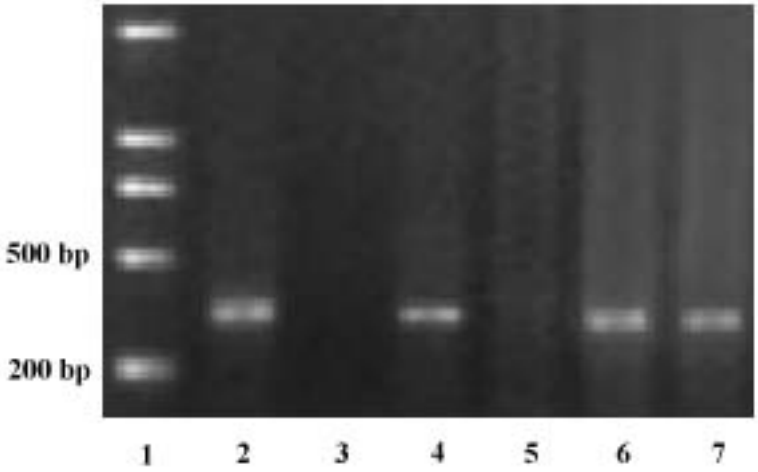


Fig.1. Electrophoresis patterns of HPV16 E7 DNA PCR products.
Lane 1: Markers； Lane 2: CaSki cell lines; Lane 3: Negative control; Lane 5: Rectal normal mucosa; Lane 4, 6, 7: Colorectal adenocarcinoma from different patients.

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