

# Detection of human papillomavirus in Chinese esophageal squamous cell carcinoma and its adjacent normal epithelium

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**Supported in part by** grants from the National Natural Science Foundation of China, No. 39925020 and from State Key Basic Research Program, No.G1998051204

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**Received:** 2002-12-22 **Accepted:** 2003-03-05

## Abstract

**AIM:** To investigate the putative role of human papillomavirus (HPV) infection in the carcinogenesis of esophageal squamous cell carcinoma in China.

**METHODS:** Twenty-three esophageal squamous cell carcinoma samples and the distal normal epithelium from Shanxi Province, and 25 more esophageal squamous cell carcinoma samples from Anyang city, two areas with a high incidence of esophageal cancer in China, were detected for the existence of HPV-16 DNA by PCR, mRNA *in situ* hybridization (ISH) and immunohistochemistry (IHC) targeting HPV-16 E6 gene.

**RESULTS:** There were approximately 64 % (31/48) patients having HPV-16 DNA in tumor samples, among them nearly two-thirds (19/31) samples were detected with mRNA expression of HPV-16 E6. However, in the normal esophageal epithelium from cancer patients, the DNA and mRNA of HPV-16 were found with much less rate: 34.7 % (8/23) and 26.1 % (6/23) respectively. In addition, at protein level detected by IHC assay, 27.1 % (13/48) tumor samples had virus oncoprotein E6 expression, while only one case of normal epithelium was found positive.

**CONCLUSION:** HPV infection, especially type 16, should be considered as a risk factor for esophageal malignancies in China.

Zhou XB, Guo M, Quan LP, Zhang W, Lu ZM, Wang QH, Ke Y, Xu NZ. Detection of human papillomavirus in Chinese esophageal squamous cell carcinoma and its adjacent normal epithelium. *World J Gastroenterol* 2003; 9(6): 1170-1173  
<http://www.wjgnet.com/1007-9327/9/1170.asp>

## INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the leading causes among Chinese cancer mortality, and the

incidence is mainly aggregated in North China, from which Henan and Shanxi Provinces are two high-incidence areas. The distinct geographical distribution suggests a dominant role of environmental factors in the etiology of this disease. Furthermore, other risk factors have been speculated, such as nutrition imbalance (lack or absence of vitamins and minerals), improper life style (cigarette smoking and consumption of pickled food), exposure to nitrosamines, during the carcinogenesis of ESCC in China<sup>[1-2]</sup>. Nevertheless, the real causes and the mechanism of ESCC have not been elucidated yet.

Human papillomavirus (HPV) as one kind of important tumor-related virus has been firmly recognized in cervical cancer. But its oncogenic role in other tumors is still disputed<sup>[3-5]</sup>. As to its role in ESCC, it was firstly suggested by Syrjanen 20 years ago, when he found the HPV infection in ESCC by pathological observation<sup>[6]</sup>. Since then, many reports regarding this topic have been published, but the HPV infection rate in ESCC varied from zero to 67 %<sup>[7,8]</sup>, depending on the specimens obtained from low- or high-risk area around the world and the methods used in each study<sup>[9-12]</sup>. In our previous study, we found that the prevalence of HPV-16 E6 and E7 genes in high incidence area was higher than that in low incidence area, detected by means of PCR and ISH, from the samples of balloon cytologic examination in Anyang area of China<sup>[13]</sup>. In order to confirm and further investigate the prevalence of HPV infection in ESCC, the tumor samples and the distal normal epithelia from Shanxi Province, another high incidence region in China, with the tumor samples from Anyang city together, were tested for the existence of HPV-16 DNA.

Based on our previous data<sup>[13]</sup>, in this study, we focused on the HPV-16 E6 gene, a major viral oncogene of high-risk HPV type. In addition to detecting its DNA and mRNA by using PCR and ISH, the E6 protein expression was simultaneously analyzed by IHC for all the samples. Furthermore the status of HPV-16 infection was compared between tumor samples and their adjacent "normal" esophageal epithelium.

## MATERIALS AND METHODS

### Clinical samples

A total of 48 primary esophageal carcinoma specimens and 23 normal samples were obtained. Among them, 25 cases were from Anyang City Cancer Hospital and 23 from Shanxi Province Cancer Hospital. Distal end of the 23 surgical samples was pathologically diagnosed as normal esophageal mucous in morphology. Both areas are high incidence region of ESCC in China. The group included 36 males and 12 females with an average age of 57.4 years.

All the samples were esophageal squamous cell carcinomas. None of the patients had radical therapy or chemotherapy before surgery. The paraffin-embedded, formaldehyde fixed samples were cut into 5  $\mu$ m slides continuously, one for H&E staining and others for DNA extraction, immunohistochemistry and ISH analysis.

### DNA extraction and PCR

The methods were as described previously<sup>[13]</sup>. Briefly, 5-10

slides were deparaffinized in xylene and graded alcohol, then the lysis buffer (300 mmol/l NaCl; 50 mmol/l Tris·HCl pH 8.0; 0.2 % SDS) was added into the tube with proteinase K (200 mg/l), and the solution was incubated at 55 °C overnight until it became clear. Then DNA was extracted using phenol/chloroform, precipitated with cold alcohol, and dissolved in ion-free water and the concentration was determined from its optical density. Quality of the extracted DNA was tested by PCR with  $\beta$ -actin primer: 5' GGC GGC ACC ACC ATG TAC CCT 3' and 5' AGG GGC CGG ACT CGT CAT ACT 3'. The usable DNA went through PCR amplification using primer: 5' -CAAGCAACAGTTACTGCGA-3' and 5' -CAACAAG-ACATACATCGACC-3' targeting HPV-16 E6 gene under conditions at 94 °C denaturing for 1 min, at 60 °C annealing for 1 min, and at 72 °C prolonging for 1 min with 30 cycles.

The PCR product was about 321 bp. The plasmid containing full of length of HPV-16 genome as template was the positive control, and the water as template was the negative control.

#### *In situ hybridization assay*

HPV-16 E6 gene by PCR from the plasmid containing full length of HPV-16 was obtained and cloned into PGEM-T easy vector (Promega). After Sal I digestion, a digoxin-labelled E6 probe was made via *in vitro* transcription with the kit (Roche, No, 1175025).

The slides were deparaffinized and hydrated in xylene and graded ethanol continuously, then were treated with 0.2 mol/L HCl for 10 min at room temperature, followed by digestion with proteinase K 100 mg/l at 37 °C for 15 min. Twenty-five to fifty ng probe was added into hybridization solution (50 % formamide, 4×SSC, 5 % dextran sulfate, 5×Denhardt's solution and 200 g/l denatured salmon sperm DNA), then the solution containing E6 probe was dropped on the slides. The hybridization reaction was completed overnight in wet-chamber at 42 °C. After this, the slides were washed by 2×SSC, 1×SSC orderly twice, 30 min each time. The anti-digoxin antibody conjugated with alkaline phosphatase was added to the samples for 30 min at 37 °C. The purple-blue ISH signals were developed by adding substrate NBT/BCIP (Roche, No, 1175041) on the slides. The slides were incubated with hybridizing solution without E6 probe as negative control, and the cervical cancer biopsies were used as positive control.

#### *Immunohistochemical staining for HPV-16 E6*

The sections were deparaffinized in xylene and hydrated in graded ethanol continuously. Then the sections were covered with 3 % hydrogen peroxide in PBS to block the endogenous peroxidase activity for 10 min. The sections were pre-treated in citrate buffer (0.01 M, pH=6.0) under microwave heating for 20 min to retrieve the antigen. Normal goat serum was added to the slides for 30 min at room temperature. After that, the sections were incubated overnight at 4 °C with mouse monoclonal primary antibody against HPV-16 E6 (Santa Cruz, sc-460#), while the negative control was incubated with PBS instead of primary antibody under the same conditions. After the slides were washed three times in PBS for 5 min each, the biotinylated goat anti-mouse secondary antibody was added for 30 min followed by the avidin-biotinylated peroxidase complex for another 30 min at room temperature. After being washed with PBS, the slides were stained with DAB, and then counterstained in haematoxylin. The cervical cancer biopsies were used as positive control.

#### *Evaluation of ISH and IHC results*

If more than 10 % of epithelial cells in one slide showed

the positive signals, the case was regarded as positive. And the data were calculated by  $\chi^2$ -test.  $P < 0.05$  was regarded as significant.

## RESULTS

### *PCR analysis*

All the extracted DNA samples showed good quality of DNA after PCR with  $\beta$ -actin primer. After PCR amplification using HPV-16 E6 specific primer, HPV infection was found in tumor patients from both regions, with an infection rate of 80 % in Anyang, and 47.8 % in Shanxi Province (Figure 1 and Table 1).

Furthermore, we detected the positive rate of 34.7 % of HPV-16 DNA in the morphologically normal epithelium adjacent to tumor tissue. But comparing the positive rate of cancer and normal samples, the difference of HPV infection in DNA level was significant (Table 1).

### *ISH and IHC assays*

To identify whether HPV-16 infection can definitely cause mRNA transcription and protein expression of E6 oncogene, we further detected these two levels by ISH and IHC.

Using digoxin-labelled HPV-16 E6 specific cRNA probe, a total of 19 samples showed positive ISH signals in the cytoplasm of cancer cells. Among them, only 2 had nuclear positive signals (Figure 1). And 6 samples from adjacent normal esophageal epithelium were positive for HPV-16.

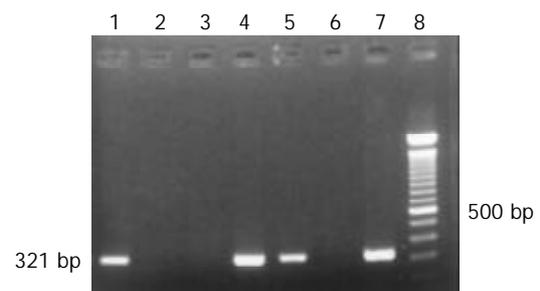
Among 48 cancer samples from both regions, 13 had HPV-16 E6 protein expression, while only one normal epithelium sample showed IHC positive signals. The difference between cancer and normal epithelia was significant ( $P < 0.05$ , Table 1).

The immunohistochemistry dark-brown signals scattered in the infected cancer cells, was similar to those mentioned before<sup>[14]</sup> (Figure 2).

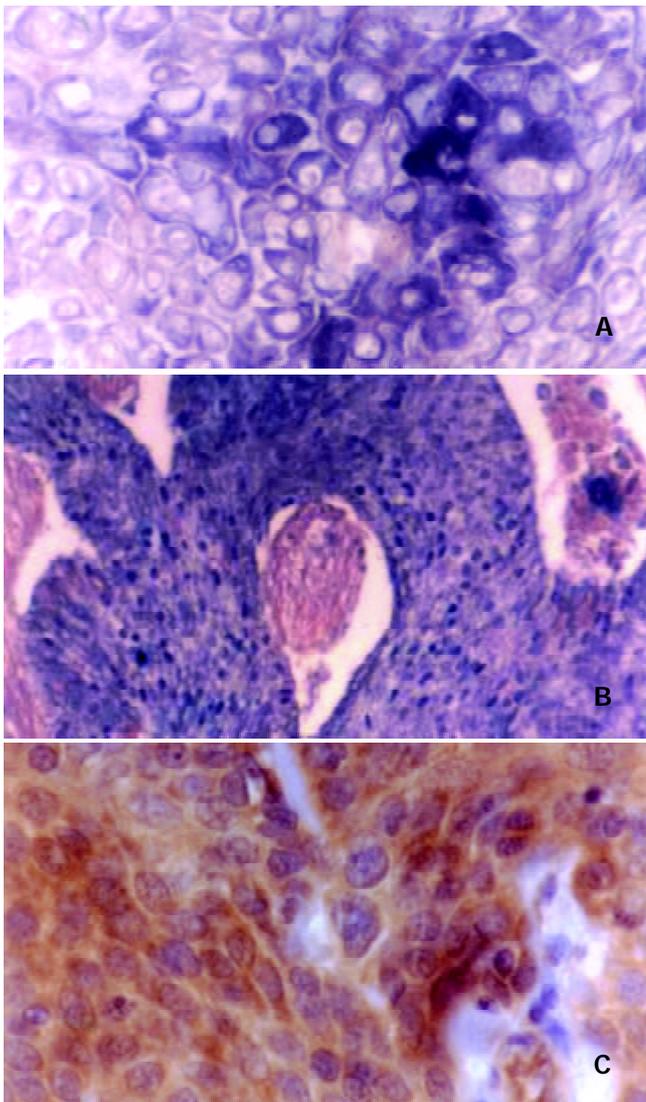
**Table 1** HPV-16 infection rate detected by PCR, ISH and IHC in normal and tumor esophageal epithelium from two high-incidence regions in China

Samples	Region	n	PCR	ISH	IHC
Normal	Shanxi	23	8(34.7%) <sup>a</sup>	6(26.1%)	1(4.3%) <sup>a</sup>
Tumor	Shanxi	23	11(47.8%)	6(26.1%)	5(21.7%)
	Anyang	25	20(80.0%)	13(52.0%)	8(32.0%)
Total		48	31(64.6%)	19(39.6%)	13(27.1%)

<sup>a</sup> $P < 0.05$ , from PCR and IHC data, HPV infection rate in normal esophageal epithelium was distinctly different from those in esophageal cancer samples.



**Figure 1** PCR results of Chinese esophageal cancer samples using HPV-16 E6 specific primer. Lane 1,2 were the positive and negative control; Lane 4,5,7 were the positive samples; Lane 3,6 were the negative samples; Lane 8 was 100 bp ladder.



**Figure 2** ISH and IHC results of tumor samples targeting HPV-16 E6 gene. A, the positive purple-blue ISH signal is mainly located in the cytoplasm of esophageal cancer cell.  $\times 200$ ; B, the positive purple-blue ISH signal is mainly located in the nuclear of the carcinoma cell.  $\times 100$ ; C, note the dark-brown IHC signals located mainly in the cytoplasm of cancer cell. SP methods, haematoxylin counterstained  $\times 200$ .

## DISCUSSION

HPV infection of the esophagus was first suggested by Syrjanen in 1982, who found that 40 % of the esophageal specimens with squamous cell carcinoma presented histological changes identical to those of condylomatous lesions in or around these carcinomas, and demonstrated the presence of HPV antigen in one case of esophageal squamous cell papilloma using immunohistochemistry. This finding was subsequently confirmed by others who demonstrated HPV antigens as well as HPV DNA in benign and malignant esophageal lesions<sup>[15-19]</sup>. But the infection rate reported in the literature varied largely, such as in India, one of Asian high ESCC regions, the detection rate could reach as high as 67 %<sup>[8]</sup>, while in some Western countries such as France<sup>[20]</sup>, Slovenia<sup>[21]</sup>, Sweden<sup>[22]</sup>, Belgium<sup>[23]</sup> and Finland<sup>[24]</sup>, the HPV infection rate is close to zero. Based on our previous study<sup>[13]</sup>, and after carefully reviewing the literature, we share the opinion with others<sup>[21,25]</sup> that HPV infection plays a much more significant role in ESCC in those regions of the world with a high prevalence of disease than in areas with a moderate or low risk<sup>[26-28]</sup>.

Besides the influence of geographic, environmental and

racial differences, the sensitivity of the detection techniques and the different methodologies should not be ignored for the variation around the world. For example, consensus L1 primers were frequently used as the proper PCR primers for its ability to detect a wide spectrum of HPV types<sup>[12,26,29]</sup>. But during virus integration into the host genome, L1 and L2 were often lost. Therefore, the detection using consensus primers against the L1 gene would likely lead to a low rate. This has been demonstrated clearly by our previous and other studies<sup>[13,25]</sup>.

HPV type 16 has been most commonly implicated in ESCC, and in addition, it is known that the E6 amplification system is retained during viral integration into the host genome<sup>[25]</sup>. In this study, we used specific primer targeting E6 gene of HPV-16 as before<sup>[13]</sup>. The presence of HPV-16 DNA was found in 65 % tumor patients from Anyang city and Shanxi Province, two high-incidence regions in ESCC in China. Furthermore, E6 mRNA expression of HPV-16 was detected in nearly two-thirds samples among those viral DNA positive patients, while the positive rate of E6 protein expression not found as high as that of mRNA, but still in more than 40 % (13/31) tumor samples, the E6 protein could be detected by IHC when HPV-16 infection occurred among those patients. From this study, it is demonstrated that HPV-16 infection of esophageal epithelium is very common within ESCC patients from Anyang city, and this observation is truly the same as within those from Shanxi Province.

Comparing the status of HPV-16 infection between some adjacent "normal" esophageal epithelium and their tumor samples, the most significant difference was E6 expression at protein level, rather than DNA and mRNA level between them (Table 1). Therefore, it is confirmed that not only existence of the viral DNA, but also the expression of E6 gene may play an important role in the carcinogenesis of ESCC in those high incidence regions.

It is well known that HPV oncogenes expressed in cervical cancer cells are involved in their transformation and immortalization, and are required for the progression towards malignancy<sup>[30-35]</sup>. In cervical cancer, the knowledge has been firmly established that HPV infection could interfere normal cell cycles by degrading tumor suppressor protein P53 and Rb and cause host genomic instability through its DNA integrating host genome randomly and increasing centrosome number. As for the exact function of HPV infection during the carcinogenesis of ESCC, it is still unclear. But in recent years, more evidences suggested the possible mechanism of high-risk HPV in transforming esophageal epithelial cells, such as induction of HPV on the activity of telomerase<sup>[36]</sup>, interaction of E6 with P53<sup>[37]</sup> the association of HPV-16 E6 with nuclear matrix<sup>[38]</sup> and others<sup>[39-40]</sup>.

In conclusion, from our previous study and this study, as well as others, HPV infection should be considered as a risk factor for ESCC, at least in high incidence area in China, and in order to further explore the role of viral DNA infection during the carcinogenesis of ESCC, more works are needed in the future.

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