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ESOPHAGEAL CANCER

Evidence of human papilloma virus infection and its epidemiology in esophageal squamous cell carcinoma

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Abstract

AIM: To look for the evidence of human papilloma virus (HPV) infection in esophageal squamous cell carcinomas (ESCC) and to investigate the potential role and epidemiology of HPV infection in the pathogenesis of esophageal carcinomas in Henan emigrants.

METHODS: Papilloma virus（PV）and HPV were determined by Ultrasensive™ S-P immunohistochemistry (IHC) and *in situ* hybridization (ISH) in esophageal carcinoma tissues (82 cases) and the normal mucosa (40 cases).

RESULTS: IHC revealed that the positive rate of PV was 75.0%, 68.18% and 72.5% respectively while the HPV (16/18-E6) positive rate was 45.0%, 36.36%, 37.5%, respectively in esophageal carcinoma tissue specimens from Henan emigrants,the local citizens and patients in Hubei Cancer Hospital. The PV and HPV (16/18-E6) were negative in all normal esophageal mucosa specimens. No correlation was found between HPV in esophageal squamous cell carcinoma tissues and in grade 1-3 esophageal squamous cell carcinoma cells. *In situ* hybridization showed that the HPV (16/18) DNA positive rate was 30.0%, 31.8%, 25.0%, respectively in the 3 groups of samples. No positive hybridization signal was found in 40 normal esophageal mucosa specimens. The positive rate of HPV (16/18) DNA in the esophageal carcinoma specimens was significantly higher than that in normal mucosa specimens ($P < 0.05$). The positive rate was not different among the 3 groups of esophageal carcinoma tissue specimens ($P > 0.05$).

CONCLUSION: HPV infection is high in esophageal carcinoma of Henan emigrants, local residents and patients in Hubei Cancer Hospital. HPV is closely related with esophageal squamous cell carcinoma. HPV infection may play an important role in esophageal squamous cell carcinoma.

Key words: Human papillomavirus; Esophageal squamous cell carcinoma; Immunohistochemistry; *in situ* hybridization

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INTRODUCTION

Chaihu area in Hubei Province, China is a high incidence area of esophageal carcinoma because almost 40 000 residents were emigrated from Henan, the highest incidence province of esophageal carcinoma incidence area. The incidence and mortality of esophageal cancer in Hubei have been increased since 1970s ($\geqslant 131/10^5$)[1]. Studies have shown that HPV plays a key role in the development of squamous cell carcinoma at various body sites, including cervix, anogenital region and oral cavity[2-4]. Esophageal squamous epithelium is one of the susceptible sites to HPV. So far, there is no report on the correlation of HPV infection and high incidence of esophageal carcinomas in Henan emigrants and its epidemiology has not been fully studied. In this study, we determined the incidence of the high-risk HPV types 16 and 18 in patients with esophageal cancer using ultrasensitive™ S-P immunohistochemistry (IHC) and in situ hybridization (ISH) and investigated the potential role and epidemiology of HPV infection in the pathogenesis of esophageal carcinoma.

MATERIALS AND METHODS

Tissue collection

Twenty specimens of esophageal carcinoma were collected from Zhongxiang Chaihu Hospital in the high-

incidence area. The average age of the patients was 58 years (range 41-58 years). Twenty patients were all Henan emigrants. Twenty-two specimens were collected from Macheng district. The mean age of patients was 55.7 years (range 33-67 years). Forty specimens were collected from Department of Pathology, Hubei Cancer Hospital. The mean age of patients was 56.9 years (range 37-75 years). The patients were all natives. All the patients were histologically diagnosed as esophageal squamous cell carcinoma.

All the samples were cut into 5-μm thick sections using Leica RM2135 microtome. H-E staining was performed before IHC and ISH. At the same time, all the samples were confirmed by pathologists and 40 normal esophageal mucosa tissue specimens were used as controls. PV and HPV (16/18-E6) antibody and Ultrasensive™ S-P immunohistochemistry kit were purchased from Maixin Biotechnology Company (Fuzhou, China). Biotinylated HPV (16/18) DNA probes and *in situ* hybridization detection systems were purchased from DAKO Company.

Immunohistochemistry

Immunohistochemistry (Ultrasensive™ S-P method) for detection of PV and HPV (16/18-E6) was performed following the manufacturer's instructions. Briefly, paraffin-embedded sections were dewaxed, antigen retrieval was performed by heating the sections in 10mM of citrate buffer (pH 6.0) for 90 s. The tissue sections were treated with 3% hydrogen peroxide in PBS containing 0.01 mol/L sodium phosphate (pH 7.2), then with 0.15 mol/L NaCl to block endogenous peroxidase and normal rabbit serum to block non-specific binding sites. Mouse monoclonal anti- HPV (16/18-E6) antibody was used as the primary antibody at a dilution of 1:100. Rabbit polyclonal anti-PV antibody was used as the primary antibody at dilution of 1:50 and 1:100, respectively. Peroxidase activity was measured with 3, 3'-diaminobenzidine. The primary antibody was absent in negative controls. Sections were counterstained with hematoxylin. The positive control sections were supplied by Maixin Bio Company.

In situ hybridization

All in situ hybridizations were performed using in situ hybridization detection systems and biotinylated HPV (16/18) DNA probes from DAKO Company. Briefly, paraffin-embedded sections were dewaxed and then digested by immersion in 0.8% pepsin solution in 0.2N HCl at 37℃ for 10 min. Following digestion, the sections were rinsed 4 times in deionized water, then immersed in 0.3% H_2O_2 for 20 min, and rinsed 5 times in deionized water. The slides were dried in air for 15 min, one drop (approximately 20 μL) of probe was applied to the sections, and covered with coverslips. The probe and HPV target DNA were denatured by placing the slides on a PCR cycler at 90℃ for 5 min. Following denaturation, slides were transferred to a pre-warmed humid chamber for hybridization at 37℃ for 60 min. Following hybridization, coverslips were removed by immersing the slides in 1×TBST at room temperature. The slides were transferred to fresh TBST bath before strigent washing at 58℃ for 30 min followed by rinsing the slides 3 times in 1×TBST, one min each. The slides were placed on a level surface and enough streptavidin-AP reagent was applied to each section to cover the tissue, incubated for 20 min at room temperature and then enough BCIP/NBT substrate solution was applied to each section to cover the tissue. The slides were incubated at room temperature for 60 min, counterstained in nuclear fast red and cover slips were mounted. Purple-blue ISH signals were observed under microscope and photos were taken for analysis. Slides with PV and HPV positive esophageal cancer tissues were used as positive controls. The hybridization solution without probe or streptavidin-AP reagent was used as a negative control.

Evaluated standard of results and statistical analysis

Without any knowledge of any patient's clinical and pathological data, all slides were evaluated independently by two pathologists. An evaluated standard of results was established corresponding to the staining intensity of positive cells: - , negative; +, 5-25% positive cells; ++, 26-50% positive cells; +++～+++++, positive cells > 50%. Statistical analyses were performed with SPSS 10.0 software and Fisher's exact probability test was used to analyze the correlation between HPV expression and clinicopathologic features of ESCC. $P<0.05$ was considered statistically significant.

RESULTS

Immunohistochemical data of PV and HPV (16/18-E6)

IHC revealed that the positive rate of PV was 75.0% (15/20), 68.18% (15/22) and 72.5% (29/40) respectively and the HPV positive rate was 45.0% (9/20), 36.36% (8/22), 37.5% (15/40) respectively in esophageal carcinoma tissues from Henan emigrants, the local citizens, and patients in Hubei Cancer Hospital. No PV and HPV were detectable in all normal esophageal mucosa tissues. Only few samples showed weak staining. No correlation was found between HPV infection in esophageal carcinoma tissues and grades of esophageal carcinoma cells. The positive rate of HPV in three groups of esophageal cancer samples was significantly higher than that in normal mucosa samples ($P < 0.01$). The positive rate was not obviously different among the three groups of esophageal carcinoma tissue samples. PV concentration at 1:50 and 1:100 showed the same positive rate (Table 1, Figures 1 A-1C).

In situ hybridization data of HPV (16/18) DNA

In situ hybridization showed that the HPV (16/18) DNA positive rate was 30.0% (6/20), 31.8% (7/22), 25.0% (10/40) respectively in esophageal carcinoma tissues from Henan emigrants, the local citizens, and patients in Hubei Cancer Hospital. Forty normal mucosa tissue specimens showed negative results (Table 2). Among the three kinds of esophageal carcinoma tissue samples, no statistically significant difference was found ($P>0.05$), while significant difference was found between normal mucosa tissues and the three kinds of esophageal carcinoma tissue samples ($P<0.05$). Positive signals of HPV (16/18) DNA were located in nuclei. Positive cells located in the center of carcinoma were in the shape of small shuttle (Figure 2A). The negative control is shown in Figure 2B.

Table 1 Expression of PV and HPV (16/18-E6) in esophageal carcinoma and normal mucosa

Origin of ESCC samples	Patients (*n*)	Positive rates of PV (%)	Positive rates of HPV (%)
Henan emigrants	20	(15/20) 75.00	(9/20) 45.00
Local residents	22	(15/22) 68.18	(8/22) 36.36
Hubei Cancer Hospital	40	(29/40) 72.50	(15/40) 37.50
Normal mucosa	40	(0/40) 0.00	(0/40) 0.00

Table 2 Expression of HPV (16/18) DNA in esophageal squamous cell carcinoma and normal mucosa tissues

Origin of ESCC samples	Patients (*n*)	-	+	+ + ~ + + + +	Positive rate (%)
Henan emigrants	20	14	3	3	30.0
Local residents	22	15	5	2	31.8
Hubei Cancer Hospital	40	30	6	4	25.0
Normal mucosa	40	40	0	0	0.00

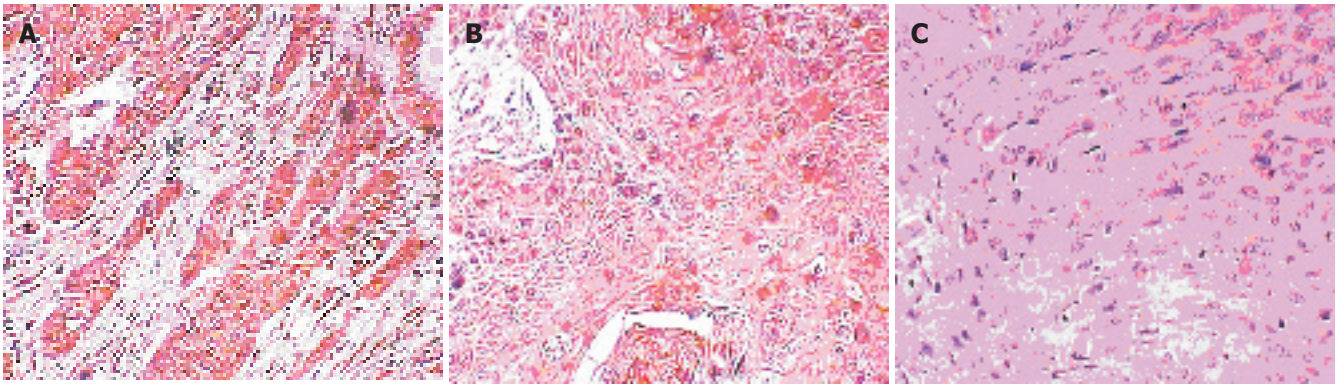

Figure 1 Positive expression of PV (**A**) and HPV(16/18-E6) (**B**) and negative expression of HPV (16/18-E6) (**C**) in esophageal carcinoma (IHC×100)

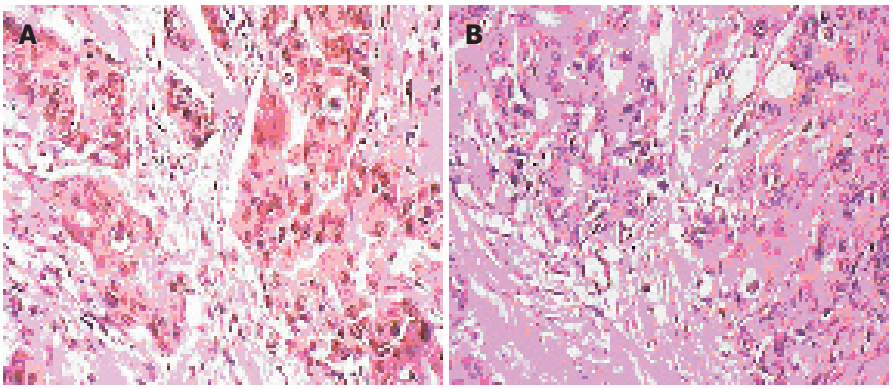

Figure 2 Positive (**A**) and negative (**B**) expression of HPV(16/18) DNA in esophageal squamous cell carcinomas (ISH×100)

DISCUSSION

Esophageal carcinoma is one of the major cancers in China. It may be closely related with heredity, environment, diet and infection with some microorganisms. One of the characteristic features of esophageal carcinoma is its variation in both geographic location and way of life. At present, it is generally believed that esophageal carcinoma is a kind of disease involving many factors. Esophageal carcinoma usually shows a typical course of carcinogenesis. Progression to malignancy during HPV-associated carcinogenesis is related to gene amplification and activation as well as high expression of many cancer genes because of mutation and deletion of cancer suppressor genes[7]. However, its mechanism has not been fully elucidated.

In 2004, approximate 1 000 questionnaires of epidemiology to Henan emigrants showed that the high incidence and mortality of esophageal carcinoma in Henan emigrants are related with environment, diet and genetic susceptibility.

Why HPV infection is related with etiology of esophageal cancer remains unclear[8]. HPV infection is first suggested as a contributory factor for the development of

esophageal cancer in 1982 by Syrjanen *et al*[9]. The presence of HPV antigen has been demonstrated by immunohistochenmical techniques[10]. Subsequently, many studies on HPV infection in esophageal cancer have been reported[11-13, 21]. However, the involvement of HPV remains controversial. Up to now, no report is available on esophageal carcinoma of Henan emigrants. In our study, the PV and HPV positive expression in esophageal carcinoma tissues was determined by Ultrasensive^{TM} S-P immunohistochemistry and *in situ* hybridization. The results showed that the positive rate in three groups of samples was high. HPV positive cells were found in the central region of tissue sections, suggesting that the positive signals (i.e. HPV DNA in the section area) are free of contamination which might come from the experiment. In immunohistochemistry, the PV and HPV positive rates were 75%, 68.18%, 72.5% and 45%, 36.36%, 37.5% respectively, while the expression rate of HPV was 30.0%, 31.8%, 25.0% respectively *in situ* hybridization in the 3 groups of samples, suggesting that HPV infection may be an integral part of a multistep process leading to esophageal cancer in high risk area. The results are consistent with other reports[14,15,20]. The positive rate of HPV detected by immunohistochemistry was higher than that by *in situ* hybridization in our study. These differences probably result from variations in the specificity and sensitivity of the analytical techniques used. *In situ* hybridization is more sensitive and specific than immunohistochemical method. Studies have generated contradictory data possibly due to the geographical location with respect to either low or high incidence areas[16,17]. In addition, variations in infection rate of HPV from the same geographical areas have been confirmed[18,19].

In conclusion, HPV infection is high in esophageal carcinoma of Henan emigrants. HPV is closely related with esophageal squamous cell carcinoma.

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