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Relationship between HPV Infection and Abnormal Expression of p53 Protein in Oral Squamous Cell Carcinoma

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Objective: *To study the relationship between human papilloma virus (HPV) infection and abnormal expression of p53 protein in oral squamous cell carcinoma (OSCC).*
Materials and Methods: *A total of 50 biopsy specimens from patients with OSCC were studied. Molecular biological and immunohistochemical methods were used to detect HPV infection in OSCC and abnormal expression of p53 protein.*
Results: *Of the 50 specimens, 14 showed HPV DNA. HPV type 16 was dominant, and one case was infected with HPV type 16 and 18. There were 9 cases of HPV infection in 24 samples with p53 overexpression, and 5 cases in 15 samples without p53 overexpression.*
Conclusions: *HPV type 16 and 18 seem to be risk factors in oral carcinoma development. However, results are greatly varied, from 5% to 95%. The cause may be due to sensitivity of detection methods and the number of samples. Regarding p53 expression, the present study shows that the HPV infection and p53 expression in oral cancer are not closely related.*
Key words: *human papilloma virus (HPV), oral cancer, p53*

Human papilloma virus (HPV) infection has been confirmed as a significant risk factor for uterine cervical carcinoma. However, its role in the development of oral carcinoma requires further study.

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The latest meta-analyses of epidemiological studies as well as the multi-centre case-control studies have confirmed that HPV is an independent risk factor for oral cancer[1,2]. The incidence of HPV infection in oral squamous cell carcinoma (OSCC) varies from 20% to 60% worldwide, and from 7.5% to 95% in China[3,4]. The cause of the high incidence of HPV infection in oral cancer in China needs further evaluation. HPV type 16 and 18 encode E6 oncoprotein, which binds to and induces degradation of the tumour suppressor protein p53. Although alcohol and tobacco use have long been considered major risk factors in oral cancer, the role that HPV plays in oral cancer requires further investigation. The purpose of the present study is: i) to determine the frequency and types of HPV in OSCC and the status of p53

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protein; ii) to find the relationship of HPV infection and p53 protein expression in OSCC; and iii) to determine whether HPV infection is a risk factor independent of alcohol and tobacco use.

Materials and Methods

Subjects

OSCC biopsies were collected from 50 patients in the Department of Oral and Maxillofacial Surgery Peking University School and Hospital of Stomatology, and kept in liquid nitrogen for HPV DNA detection. Of these, 39 were evaluated by immunohistochemistry staining against p53 antibody. Permission was obtained from the patients for part of their operation sample to be used for scientific investigation according to the rules of Peking University Health Science Center IRB.

Methods

DNA Buffer, Taq DNA polymerase (Promega), dNTPs (Pharmacia), 100 bp DNA standard (GIBCOBRL), Thermal Cycler 90AD (China Academy of Science Institute of Heredity), p53 monoantibody (DAKO), biotinylation second antibody and streptavitin (ZYMED), DAB (SIGMA) HPV general primer: MY11: 5'-GCC (AC)C AGG G(AT) CAT AA(CT) AAT GG-3', MY09: 5'-CGT CC(AC) A(AG) (AG)G GA(AT) ACT GAT C-3'; HPV16 specific primer: 5'-AGC TCA GAG GAG GAG GAT GA-3', 5'-GGT TTC TGA GAA ACA GTG GG-3'; HPV18 specific primer: 5'- GAG CCC CAA AAT GAA ATT CC-3', 5'-CAA AGG ACA GGG TGT TCA GA-3'. HPV general primer PCR products are 450 bp; HPV16 primer PCR products are 203 bp; HPV18 primer PCR products are 244 bp.

DNA preparation

The oral cancer tissues kept in the liquid nitrogen were washed in DNA buffer (100 mmol/l Tris, 40 mmol/l EDTA, 100 mmol/l NaCl, 1% SDS, pH 8.0), and cut into small pieces. Homogenate was obtained using 1 g of tissue in 600 µl of DNA buffer and was transferred into an EP tube. Proteinase K was added to get a final concentration of 0.5 µg/µl, and this was kept in a 37 ºC water bath overnight. For DNA purification, the homogenate was mixed with an equal volume of phenol and centrifuged for 10 min at 10,000 rpm. The supernatant was transferred to a fresh tube and mixed with an equal volume of phenol/chloroform, centrifuged for 2 min at 10,000 rpm, and the supernatant was removed to a fresh tube. One tenth of the volume of 3 mol/l NaAc (pH 4.6) and 2.5 volumes of pre-cooled ethanol (-20 ºC) were added, and this was kept at -20 ºC for 20 min. The mixture was centrifuged for 20 min at 12,000 rpm, the supernatant was removed, and the pellet was rinsed with 1 ml of 70% precooled (-20 ºC) ethanol twice. The DNA pellet was dried in the air for 5 min and then dissolved in 50 µl ddH_2O. The DNA was diluted to 0.5 µg/µl, and stored at -70 ºC.

PCR performance

Each PCR reaction mixture consisted of 10 mmol/l of Tris-HCL (pH 8.3), 50 mmol/l of KCL, 1.5 mmol/l of $MgCl_2$, 0.2 mmol/l of dNTPs, 2.5 µg of primers and 0.5 µg of template DNA. The total reaction volume was 25 µl. Pre-denature was carried out at 95 ºC for 5 min, and then 0.5 units of Taq DNA polymerase was added into per reaction tube, and mixed completely. The PCR procedures were as follows: general primers, HPV 16 primers and HPV 18 primers were denatured at 95 ºC for 60 s, 55 s, and 45 s respectively, annealed at 50 ºC for 45 s, and extended at 72 ºC for 60 s. All the extensions were carried out at 72 ºC for 7 min after 35 cycles. The size of PCR products were checked by 12% PAGE electrophoresis at 40 V for 2 hours and 30 min for general primer, and 1 hour and 30 min for HPV 16 and 18 primer. The gel was stained in 0.5 µg/ml EB solution for 10 min. The pSHPV-18 plasmid contained HPV18 DNA and pointed condyloma DNA was employed as positive control. The ddH_2O was used to take the place of template DNA as blank control. Non-HPV infection normal oral mucosa DNA was used as negative control.

Immunohistochemistry stain with p53 antibody

The oral cancer tissues were fixed in 10% formalin and embedded with paraffin, cut to 4 µm sections, and deparaffinised at 58 ºC overnight. For use, the sections were hydrated in gradient ethanol, fixed with 3% H_2O_2-methanol for 20 min; the sections were rinsed with Tris buffer three times. Antigen retrieval was performed by heating the section in a 700 W microwave oven for 10 min, and washing the slice with Tris buffer three times after the slice had cooled to room temperature. Then the p53 antibody (1:50 dilution) was added and kept in a wet box for 1 hour, further washed with Tris buffer three times. The second antibody (1:200 dilution) was added, and it was again kept in a wet box for 30 min, followed by washing in Tris buffer three times. Streptavidin (1:200 dilution) was added and the slice was kept in the wet box for 30 min, followed by washing with Tris buffer three times. Finally, DAB colouration was performed for 5-10 min at room temperature (10 mg DAB dissolved in 50 ml Tris buffer, 7-14 µl 30% H_2O_2). The reaction was stopped with running water, and counterstaining was done with haematoxylin.

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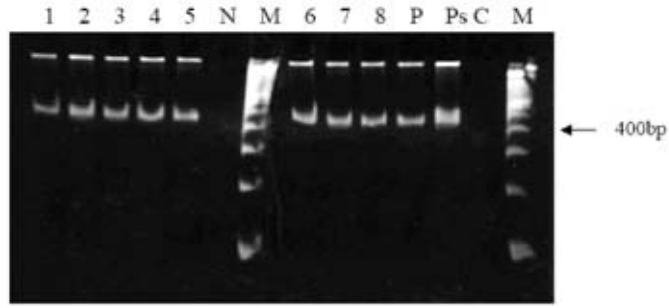

Fig 1 Electrophorotype of PCR products amplified with general HPV primer. PCR products size 460 bp was amplified with general primer. M, 100 bp DNA standard; Ps, psHPV18 plasmid DNA; P, DNA of pointed wart; C, empty control; N, human normal oral mucosa DNA; 1–8: HPV positive samples.

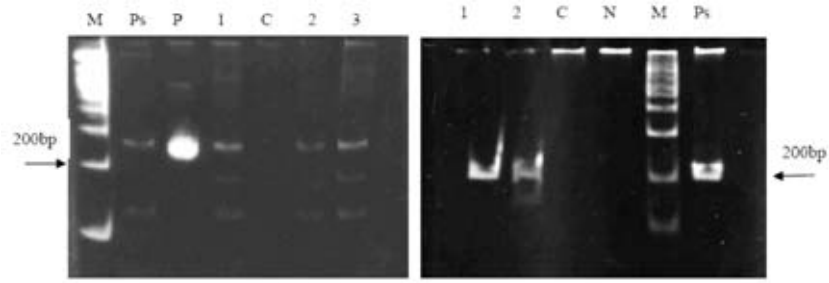

Fig 2 Electrophorotype of PCR products amplified with HPV 16 and 18 primer. PCR products size 203 bp (right) and 244 bp (left) were amplified with HPV16 and 18 primer. M, 100 bp DNA standard; Ps, psHPV18 plasmid DNA; C, empty control; N, human normal oral mucosa DNA; 1–3, HPV positive samples.

TABLE 1 Frequency of HPV DNA infection in oral cancer tissue (n = 50)

	High risk HPV type HPV16	HPV18	HPV16+18	Total in high-risk type HPV
Positive	10	4	1	14
Negative	40	46	49	0
Frequency (%)	20	8	2	28

Results

Detection of HPV infection in oral cancer tissue

PCR assay revealed that HPV DNA was present in 14 of 50 (28%) oral squamous cell carcinoma samples. The HPV type 16 DNA was predominant in 13 of HPV infected OSCC (92.9%). One of the samples had been infected by both HPV type16 and 18 DNA (Table 1, Figs 1 and 2).

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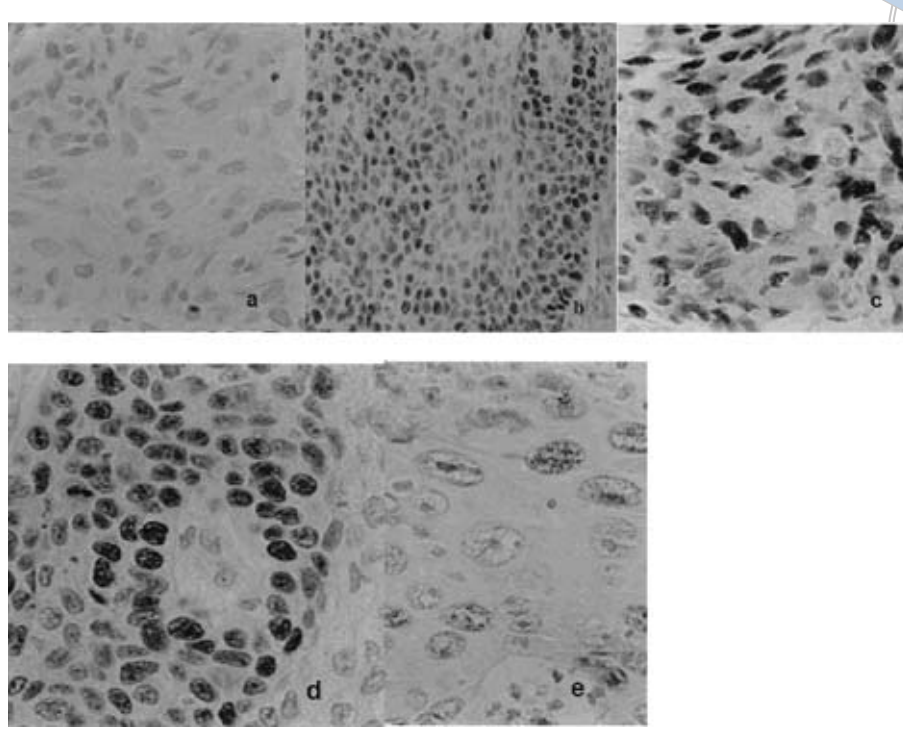

Fig 3 Oral cancer tissues stained by immunohistochemistry method against p53 antibody. The nuclei stained in brown colour were p53 positive (b–e) and in light blue were p53 negative (a); vacuolar degeneration can be seen in some tissues (e). Original magnification 40x.

TABLE 2 Relationship of HPV infection and p53 abnormal expression in oral cancer

	HPV-positive	HPV-negative	Total	Frequency of HPV-positive (%)
p53-positive	9	15	24	37.5*
p53-negative	5	10	15	33.3*
No detection of p53	1	10	11	
Total	15	35	50	

* no significant difference (p = 0.79) between p53-positive with HPV infection and p53-negative with HPV infection in oral cancer.

Exceptional expression of p53 protein in oral cancer
The expression of p53 protein was as high as 61.5% (24/39) (Fig 3).

Relationship of HPV infection and p53 expression
The frequency of HPV infection was 37.5% (9/24) among samples with p53 overexpression and 33.3% (5/15) in samples without p53 overexpression. There was no significant difference in the high-risk type of HPV infection between samples with and without p53 overexpression by χ^2 test ($p = 0.79$; Table 2). The correlativity of HPV infection and p53 exceptional expression in oral cancer was not closely related ($r < 1$). There were 20% (10/50) of oral cancer tissues that had either no HPV infection or no p53 overexpression.

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Discussion

The incidence of oral cancer has recently increased worldwide. Tobacco use and alcohol consumption are accepted oral cancer risk factors. HPV infection is another possible risk factor in oral carcinogenesis according to epidemiological study. However, the frequency of HPV infection in squamous cell carcinoma has varied relatively greatly among studies[3,4]. The present study found that the frequency of HPV infection in OSCC was 28% (14/50), most of which were high-risk type HPV (13/14). HPV type 16 was predominant among the samples, in agreement with most previous studies[5,6,7], but in contrast to a study by Shin et al[8], in which HPV type 18 was predominant in Korean oral cancer tissue. Nagpal et al[9] studied a total of 110 cases of oral cancer, in patients highly addicted to betel quid and tobacco chewing. A total of 37 patients (33.6%) showed the presence of HPV, among them the presence of HPV16, 18 and 16/18 co-infection is 22.7%, 14.5% and 10%, respectively. In a study by Wang et al[2], a wide range in the frequency of HPV infection in OSCC was reported. This may be due to the sensitivity of detection methods, and the number of detected samples. Therefore, the frequency of HPV infection and the relationship between HPV infection and p53 abnormal expression needs further study in the Chinese population.

p53 inactivation is a major mechanism of HPV-related carcinogenesis in the oral cavity and cavum nasopharyngeum. Polymorphism at p53 codon 72 may play a role in HPV infection. The study by Nagpal et al[9] reported Arg/Arg genotype to be more susceptible to HPV infection in OSCC patients in Eastern India. In the present study, HPV infection in OSCC was not correlated with p53 oncoprotein overexpression. Therefore we suggest that there is no relationship between HPV infection and p53 oncoprotein overexpression.

In the present study, 10 samples had neither HPV infection nor p53 overexpression. Therefore, it could be proposed that 80% of oral cancers are related with HPV infection and/or p53 abnormal expression, and 20% are caused by other factors.

HPV vaccination in cervical cancer has developed quickly. One prophylactic quadrivalent vaccine using L1 virus-like particles (VLP) of HPV6, 11, 16 and 18 has been available in Europe since the end of 2006 and it is expected that a second bivalent vaccine containing VLPs of HPV16 and HPV18 will become available in 2007. It offers HPV-naive women a very high level of protection against HPV persistent infection and cervical intra-epithelial lesions associated with the types included in the vaccine[10,11]. Currently, studies have shown only that HPV infections are associated with oral cancer, although the frequency of HPV infection in oral cancer was not as high as in cervical cancer. HPV vaccine therapy may also be useful to head and neck cancer patients.

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