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Human papillomaviruses in oral squamous cell carcinoma and pre-cancerous lesions detected by PCR-based gene-chip array

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Abstract. Human papillomavirus (HPV) infection is a significant risk factor for uterine cervical carcinoma. Many studies have also demonstrated the presence of HPV in oral epithelia tissue, but the role of HPV infection in oral squamous cell carcinoma (OSCC) is still controversial. The aim of this study was to determine the frequency and type of HPV in OSCC and oral pre-cancerous lesions. DNA samples were collected by cytobrushing from 51 patients with OSCC, 46 with oral pre-cancerous lesions and 90 normal controls. Nested polymerase chain reaction and gene-chip arrays were used to identify the HPV types in the samples. In pre-cancerous lesions, there was a higher frequency of HPV of any type (14/46, OR = 2.844, CI = 1.186–6.816, $P = 0.0216$) and of low-risk HPV types (9/46, OR = 5.529, CI = 1.597–19.14, $P = 0.0096$) than in control samples. The prevalence of high-risk types was significantly higher in OSCC than in control lesions (11/51 vs 8/90, OR = 2.819, CI = 1.051–7.558, $P = 0.0420$) but this was not the case for HPV of any type (13/51 vs 12/90, OR = 2.244, CI = 0.9266–5.337, $P = 0.1066$). High-risk HPV types are prevalent in OSCC and may play a role in its progression, while low-risk types are associated with oral pre-cancerous lesions.

Key words: human papillomavirus; genotype; cytobrush; gene-chip; mouth neoplasm.

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Squamous cell carcinoma is the most common malignant neoplasm of the oral mucosa, representing more than 90% of these malignant tumours. In Taiwan, oral cancer ranks as the seventh most prevalent cancer in both sexes and accounts for the fourth most common cancer in males[3]. Known risk factors for oral squamous cell carcinoma (OSCC) are long-term tobacco, alcohol and betel nut use, radiation, viruses, and chronic irritants. While many individuals are exposed to these risk factors, only a small proportion develops OSCC. This suggests that other factors may play a role in oral carcinogenesis.

Human papillomaviruses (HPV) are small, non-enveloped, icosahedral, epitheliotropic DNA viruses. To date more than 70 different HPV genotypes have been cloned and characterized[6]. HPV infection is associated with a wide spectrum of epithelial lesions, most of which are benign hyperplasia. A subgroup of HPVs is associated with lesions that have a propensity to undergo carcinogenesis. These are considered high-risk HPVs, including types 16, 18, 31, 33, 35, 39, 45 and 52[1,12]. These types are known to play an important role in epithelial carcinomas of the uterine cervix[6]. A high-risk

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virus may become integrated into the host genome where it participates in oncogenesis. After integration, two viral genes, E6 and E7, are consistently expressed. The E6 and E7 genes of HPV encode oncoproteins that target the p53 and retinoblastoma tumour suppressor genes, resulting in loss of control over the cell cycle[1].

The oral cavity, like the uterine cervix, is covered by squamous epithelium with or without slight keratinization and is continuously exposed to the external environment. Many previous studies have demonstrated the presence of HPV in oral epithelial tissue[4,5,7–25], but the relationship of HPV to OSCC remains unclear because of the difficulty in interpreting the variations in reported prevalence of HPV in this condition. This study was designed to investigate the prevalence and typing of HPV infection in patients with both cancerous and pre-cancerous oral mucosal lesions, and to compare these with findings from a group of normal control subjects without oral mucosal disease.

Materials and methods

Patients and specimens

A total of 97 patients provided specimens for this study: 51 with OSCC (48 men and 3 women, age range 33–71 years) and 46 with precancerous lesions (45 men and 1 woman, age range 20 to 74 years), including leukoplakia, oral submucous fibrosis and verrucous hyperplasia. The diagnosis in all cases was based on histological examination of haematoxylin & eosin-stained tissue sections. All patients underwent total excision of their lesions at the Department of Oral and Maxillofacial Surgery, Mackay Memorial Hospital, Taipei, Taiwan, during January 2002 to May 2004. In addition, 90 specimens of normal oral mucosa were obtained from 90 normal subjects (52 men and 38 women, range 15 to 75 years) seen for extraction of impacted permanent lower third molars during January 2002 to January 2004. These constituted the normal control samples.

Cytological specimens were obtained from the lesions of each subject before surgery. The brush was held against the mucosa of the lesion and rotated 10 full turns. A similar procedure was performed to sample the normal mucosa of the control subjects. The brush was immediately placed in HBSS solution and kept at 4 °C until further analysis. Of the 51 cases of OSCC, 10 (20%) involved the tongue, 27 (53%) the buccal mucosa, 10 (20%) the gingiva, 2 (4%) the lips and 2 (4%) the floor of the mouth. The histological types included 4 (8%) verrucous, 23 (45%) well differentiated, 20 (39%) moderately differentiated and 4 (8%) poorly differentiated tumours.

This study was approved by the Human Research Review Committee of Mackay Memorial Hospital, and all patients gave informed consent.

DNA extraction

The exfoliated cells from the cytology brush were vortexed for 30 s in an Eppendorf centrifuge tube containing 1ml of polymerase chain reaction (PBS), pelleted by centrifugation at 1500 rpm for 5 min at 20 °C, washed three times with PBS, resuspended to a final concentration of 10^6 cell per ml in proteinase K digestion buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.5% Tween-20, 400 μg/ml of proteinase K), and incubated at 94 °C for 1 h. Standardized phenol-chloroform extraction and ethanol precipitation were used for DNA purification, and the purified DNA was resuspended in sterile distilled water. To control the quality and quantity of the isolated DNA, a 300-base-pair sequence of the β-actin gene was amplified by PCR as an internal control. The PCR product was electrophoresed on 2% agarose gel and stained with ethidium bromide.

HPV detection by nested PCR

Each PCR amplification reaction was carried out in a total volume of 50 μl containing the PCR master mixture (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 800 μM of each dNTP; 10 pM of each primer set; 1.25 U/L of AmpliTaq Gold DNA polymerase; Applied Biosystems, CA, USA). An optimal $MgCl_2$ concentration of 2.0 mM was determined to obtain specific and efficient amplification. Before amplification, the reaction mixture was treated with 1 U of uracil-N glycosylase (Roche Diagnostics, Mannheim, Germany) to prevent DNA carry-over and with 0.2 μg of TaqStart™ antibody (Clontech, Franklin Lakes, NJ, USA) to carry out a hot-start PCR.

Each PCR was carried out in the Geneamp PCR System 9700 (Applied Biosystems) with the first denaturation step at 94 °C for 3 min and the final extension step at 72 °C for 5 min. The conditions and number of denaturation–annealing–extension cycles were different for each set of primers. General consensus primers

Table 1. Human papillomavirus sequence primers used for polymerase chain reaction

Primer	Sequence
MY11	5′ GCM CAG GGW CAT AAY AAT GG
GP5+	5′ TTT GTT ACT GTG GTA GAT AC
GP6+	5′ GAA AAA TAA ACT GTA AAT CA

Table 2. HPV-positive cases in the OSCC group

Case no.	Sex	Age	TNM	Stage	Location	Differention	HPV type	Risk group	Betel nut chewing	Smoking	Alcohol
1	M	50	T4N0M0	IV	Tongue	Verrucous	33, 52	H	+	+	+
2	F	46	T2N0M0	II	Tongue	Moderate	16	H	No	No	No
3	M	61	T4N0M0	IV	Mouth floor	Well	18, 53	H	+	+	+
4	M	65	T3N0M0	III	Gingiva	Moderate	39	H	+	+	+
5	M	30	T2N0M0	IV	Buccal	Well	16	H	+	+	+
6	M	56	T3N1M0	III	Gingiva	Moderate	16	H	+	+	+
7	M	35	T2N0M0	II	Tongue	Moderate	72	L	+	+	+
8	M	49	T4N1M0	IV	Gingiva	Well	66	L	No	No	+
9	M	57	T2N0M0	II	Buccal	Well	52	H	+	+	+
10	M	34	T2N0M0	II	Buccal	Moderate	11, 18	H	+	+	+
11	M	42	T2N0M0	II	Buccal	Moderate	58, 16	H	+	+	+
12	M	41	T4N0M0	IV	Lip	Moderate	11, 16	H	+	+	+
13	M	66	T4N3M0	IV	Gingiva	Moderate	52	H	+	+	+

Table 3. HPV-positive cases in the pre-malignance group

Case no.	Sex	Age	Diagnosis	Dysplasia	HPV type	Risk group	Betel nut chewing	Smoking	Alcohol
1	M	33	Leukoplakia	No	CP8304	L	+	+	+
2	M	58	Verrucous hyperplasia	No	58	L	+	+	+
3	M	58	Verrucous hyperplasia	No	18, 31	H	+	+	+
4	M	41	Oral submucous fibrosis	No	11	L	+	+	+
5	M	43	Acanthosis	No	16	H	+	+	+
6	M	45	Epithelium hyperplasia	No	11, 16	H	+	+	+
7	M	41	Acanthosis, epithelium hyperplasia	No	16,66	H	+	+	+
8	M	50	Squamous papilloma	No	MM8, CP8304	L	+	+	+
9	M	26	Leukoplakia	No	68, 53	L	+	+	+
10	M	43	Oral submucous fibrosis	No	56	L	+	+	+
11	M	35	Squamous hyperplasia	No	53	L	+	+	+
12	M	29	Oral submucous fibrosis	No	42	L	+	+	+
13	M	41	Acanthosis, epithelium hyperplasia	No	44	L	+	+	+
14	M	52	Leukoplakia	No	52	H	+	+	+

MY11/GP6+ (Table 1) were used for the first PCR round to amplify the corresponding part of the HPV L1 gene. A nested PCR was then carried out with the primers GP5+/GP6+ (Table 1) according to previously published protocols[5].

HPV genotyping by gene chip

Fifteen microliters of the amplified products were hybridized with a revert-blot of the HPV gene chip (HPV Blot®, King Car, Taipei, Taiwan) which detects 39 genotypes of HPV DNA (6, 11, 16, 18, 26, 31, 32, 33, 35, 37, 39, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 66, 67, 68, 69, 70, 72, 74, 82, CP8061, CP8304, L1AE5, MM4, MM7 and MM8) in a single hybridization process. Briefly, the HPV chip membrane was equilibrated with 2× standard saline citrate (SCC), then prehybridized using a prehybridization buffer (2× SCC, 0.5% blocking reagent, 5% dextran sulfate and 0.1% sodium dodecyl sulfate (SDS)) containing denatured salmon sperm DNA (50 mg/L) by shaking at 35 °C for 30 min, followed by hybridization with 500 μl of hybridization buffer (2× SSC, 0.5% blocking reagent, 5% dextran sulfate, 0.1% SDS and 50 mg/L denatured salmon sperm DNA) containing denatured amplimers (15 μl) by shaking at 35 °C for at least 3 h. The chip was washed once in washing buffer-I (2× SSC and 0.1% SDS) for 5 min at room temperature and then twice in washing buffer-II (0.2× SSC and 0.1% SDS) for 5 min at 35 °C. Following this stringent washing, the chip was equilibrated with buffer-I (1× PBS pH 7.4, 0.05% polysorbate20, 0.1% SDS) by shaking twice at room temperature for 5 min. It was then incubated in 500 μl of buffer-II (1× PBS pH 7.4, 0.05% polysorbate20, 0.1% SDS, 0.5% blocking reagent) containing Avidex-APTM (alkaline phosphatase conjugated and biotinylated antibodies, 1:1000 dilution; Applied Biosystems) for 40 min. After alkaline phosphatase conjugation, the chip was washed in buffer-I and rinsed with buffer-III (0.1 mol/L HCl) for 5 min. Then, 70 μL of substrate nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate was added and incubated for 30 min at room temperature. The reaction was stopped by aspiration of the substrate solution and addition of distilled water.

After drying, the chips were assayed by genotype scanning software (HPV-EasyScan, Taiwan Molecular Diagnostics Laboratories Ltd, Taipei, Taiwan) for the HPV types on the chip. The results were rechecked by direct vision to insure accurate calibration. As a duplicate test, each independent PCR product was hybridized with a single gene-chip and the results compared with those from the gene-chip array. If there was a discrepancy between the two results, PCR using HPV type-specific primers was performed to confirm the result. Specimens hybridizing with the generic probe but not with any of the type-specific probes were considered

Table 4. Correlation between HPV positivity and clinicopathologic date in OSCC group

	HPV positive ($n = 13$)	HPV negative ($n = 38$)	Statistically significant?
Sex			
Male	12	36	
Female	1	2	
Location			No ($P = 0.1164$)
Buccal	4	22	
Other	9	16	
T status			No
T1	0	3	
T2	6	5	
T3	2	4	
T4	5	26	
N status			No ($P = 0.5061$)
N0	10	26	
N+(N1, N2, N3)	3	15	
Stage			No ($P = 0.1466$)
Early (stage I, II)	6	8	
Late (stage III, IV)	7	30	
Differentiation			No
Verrucous	0	3	
Well	5	19	
Moderate	8	12	
Poor	0	4	
Habit			
Smoking	12	37	
Alcohol drinking	12	37	
Betel nut chewing	13	37	

Table 5. Correlation between HPV positivity and clinicopathologic data in pre-malignant group

	HPV positive ($n = 14$)	HPV negative ($n = 32$)	Statistically significant?
Sex			
Male	14	31	
Female	0	1	
Dysplasia			No ($P = 0.09$)
Positive	0	2	
Negative	14	30	
Personal habits			
Smoking	14	31	
Alcohol drinking	14	30	
Betel nut chewing	14	32	

Table 6. Prevalence of HPV in OSCC and control samples

HPV type	OSCC ($n = 51$)	Controls ($n = 90$)	OR	95% CI	P value
All	13 (25.49%)	12 (13.33%)	2.224	0.9266–5.337	0.1066
High risk	11 (21.57%)	8 (8.89%)	2.819	1.051–7.558	0.0420*
Low risk	2 (3.92%)	4 (4.44%)	0.8776	0.1550–4.968	1.000

* Statistically significant.

to harbor unidentified an HPV type. This methodology, including the cytology brushing and a nested PCR-based gene-chip assay, has been shown to be reliable for determining HPV types[2,5,9].

Statistical analysis

Rates of HPV positivity were compared between the three groups (OCSS, pre-cancerous lesions and normal controls) by Fisher's exact test. The same test was used to compare rates of high-risk and low-risk HPV types in the three groups. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. Results were considered significant if the P value was less than 0.05.

Results

Amplification of the human β-actin gene in all DNA samples indicated that sufficient DNA was present for the amplification of HPV sequences. To rule out the possibility of contamination and PCR artefacts, at least three negative control samples containing all reaction components except template DNA were included for each round of PCR amplification. No PCR product was detected in the first or second round of amplification of the negative control samples, indicating the absence of contamination.

The data obtained using the nested PCR-based gene-chip method to determine multiple HPV types in OSCCs, oral pre-cancer and control samples are shown in Tables 2 and 3. The presence of HPV DNA in OSCC was not associated with personal habit, tumour location, clinical staging or histological type (Table 4). It was also not associated with personal habit and level of dysplasia in the pre-cancerous group (Table 5). The rates of HPV positivity, ORs and 95% CI for samples from OSCC and pre-cancerous lesions compared to control subjects are shown in Tables 6–8. HPV DNA was detected more frequently in pre-cancerous lesions (14/46, 30%) than in controls (12/90, 13%; $P = 0.0216$). The frequency was also higher in OSCC (13/51, 25%) than in control samples but did not reach a clear statistical difference ($P = 0.106$).

In HPV-positive OSCC, high-risk types were significantly more frequent than in control samples ($P = 0.0420$). Low-risk types were significantly more common in pre-cancerous lesions than in OSCC ($P = 0.0227$) and control samples ($P = 0.0096$). The prevalence of high-risk types did not differ significantly between pre-cancerous and control specimens.

Discussion

The discrepancy in the detection of HPV between different studies may be due to differences in the detection methods used and the source of the tissue samples. According to a review by Miller et al.[12,13], assays considered to be of low sensitivity include immunoperoxidase, immunofluorescence and *in situ* hybridization. Moderate sensitivity is provided by Southern-blot and dot-blot hybridization, while high sensitivity assays use PCR.

The study presented by Chang et al.[5] which also used the similar methods to determine the prevalence of HPV in a Taiwanese OSCC population, revealed a higher prevalence than the present study. This may be due to the difference in sampling. They used a formalin-fixed, paraffin-embedded tissue block obtained from surgical procedure and pathological processing; this has more chance of contamination before DNA extraction then a fresh sample obtained by cytobrushing.

The reviews by Miller et al. report detection of HPV DNA in 46.5% of OSCC, 22.2% of benign lesions and 10.0% of normal samples[12,13]. By comparison, the present data indicate HBV DNA in 26% of OSCC lesions, 30% of pre-cancerous lesions and 13% of normal samples. Factors contributing to differences from previously published results may include the study sample size and ethnic or regional variation, as well as methodology. The use of fresh specimens obtained by cytobrushing in a nested PCR-based gene-chip assay is non-invasive and easy to perform, has high sensitivity, and appears to be a reliable method for investigating the prevalence of HPV in oral mucosal lesions.

While the overall prevalence of HPV DNA in OSCC samples was not statistically higher than in control samples, there was a significantly higher preva-

Table 7. Prevalence of HPV in oral pre-cancerous lesions and control samples

HPV type	Pre-cancerous lesions ($n = 46$)	Controls ($n = 90$)	OR	95% CI	P value
All	14 (30.43%)	12 (13.33%)	2.844	1.186–6.816	0.0216*
High risk	5 (10.87%)	8 (8.89%)	1.25	0.3845–4.063	0.7618
Low risk	9 (19.57%)	4 (4.44%)	5.529	1.597–19.14	0.0096*

* Statistically significant.

Table 8. Prevalence of HPV in OSCC and oral pre-cancerous lesions

HPV type	OSCC ($n = 51$)	Pre-cancerous lesions ($n = 46$)	OR	95% CI	P value
All	13 (25.49%)	14 (30.43%)	0.7820	0.3212–1.903	0.6534
High risk	11 (21.57%)	5 (10.87%)	2.255	0.7186–7.077	0.1808
Low risk	2 (3.92%)	9 (19.57%)	0.1678	0.03419–0.82369	0.0227*

* Statistically significant.

lence of high-risk HPV types in OSCC. The study by Chang et al.[5] reported a similar result especially in the non-oral habit-associated patients, although not including the pre-cancerous group. The prevalence of HPV, especially high-risk types, in the overall OSCC group was higher but in the oral habit-associated group was similar to the control samples, as in the present study. In contrast, the pre-cancerous benign lesions had a higher prevalence of all HPV types than did controls. But of even more interest is the significantly higher prevalence in benign lesions of low-risk HPV types than in either control or OSCC samples. These findings of this and the previous study[5] support the hypothesis that specific HPV genotypes may be important in malignant transformation. High-risk HPV genotypes infecting the oral epithelium appear to have the potential to propagate new malignancies. The low-risk genotypes may in fact retard the malignant transformation of pre-cancerous lesions, even though the environmental risk factors and personal habits such as smoking, alcohol drinking and betel nut chewing are similar for both malignant and benign lesions.

As with most cancers, OSCC is believed to develop by a multi-step process of cumulative gene damage in oral epithelial cells[23]. Previous studies with *in situ* hybridization have demonstrated that HPV DNA localizes to the nuclei in the spinous and basal cell layer of the epithelium, with HPV-positive cells located in the center of the tumour nest[14,15,18,23]. As noted above, HPV-DNA integration into the human genome may be associated with the expression of oncogenes and the down-regulation of tumour suppressor genes in the early stage of OSCC carcinogenesis[1]. The specific type of infecting HPV may influence malignant progression in the host cell, with high-risk types accelerating the progression and low-risk types retarding it. This would account for the type prevalences in this study. The type of HPV involved may be an important prognostic factor. Schwartz et al.[20] and Gillison et al.[8] have suggested that HPV DNA is a strong, independent predictor of outcome in oral cancer, even in advanced disease. In conclusion, the high-risk HPV types may play a role in OSCC, while low-risk types are more likely to be associated with pre-cancerous lesions. Further investigation at the molecular level of the mechanisms involved may eventually lead to clinically useful methods for diagnosis, prognosis and chemoprevention.

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