INCIDENCE OF LOW RISK HUMAN PAPILLOMAVIRUS IN ORAL CANCER: A REAL TIME PCR STUDY ON 278 PATIENTS

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Squamous cell carcinoma is the most frequent malignant tumour of the oral cavity. It is widely known that tobacco and alcohol consumption are the major causes of the development of oral squamous cell carcinoma (OSCC). The human papilloma virus infection has also been postulated as a risk factor for squamous cell carcinoma, although conflicting results have been reported. The aim of this study is to evaluate the presence of high-risk and low-risk type human papillomavirus in a large sample of squamous cell carcinoma limited to the oral cavity by means of quantitative real-time polymerase chain reaction. Data were obtained from 278 squamous cell carcinoma limited to oral cavity proper. Sequencing revealed that 5 samples were positive for HPV type 16, 5 for HPV type 11, and 1 for HPV type 6. Human papillomavirus 11 was detected in 5 tumours out of the 278 examined. The prevalence rate for Human papillomavirus 11 was 1.8% (C.I. 0.7-3.9). The matched case-controls analysis indicated that the prevalence among controls did not significantly differ with respect to cases and that Human papillomavirus 11 alone did not correlate with squamous cell carcinoma.

Oral squamous cell carcinoma (OSCC) is the most frequently malignant tumor of the oral cavity with about 30,000 new cases and 8,000 related deaths per year in the United States (1).

The most important risk factors for this cancer are certainly the consumption of alcohol and tobacco, although other factors such as genetic susceptibility, diet and infection by viral agents appear to play a synergic role in the development of the disease (2).

In 1983, human papilloma virus (HPV) was first described as a factor involved in the development of OSCC, by Syrjänen (3). From then, the presence of different types of HPV has been detected in oral cancers with average prevalence rates ranging from 12 to 70% (4).

These conflicting results could be due to the differences among the detection methods (polymerase chain reaction, enzyme-linked immunosorbent assay and immunohistochemistry), epidemiology, anatomical locations, sample size (5).

HPV types have been identified and classified as high or low -risk (HR and LR). LR-HPV (i.e. HPV types 2, 4, 6, 11, 13, 32) are responsible for benign skin and mucosal lesions (condylomas, squamous cell papillomas), while HR-HPV (i.e. 16, 18, 31, 33, 35, 58) are related to malignant lesions (cervical intra-epithelial neoplasms, cervical, penile and vulvar carcinomas, giant condylomas) (6). The hypothesis of a role for HPV in the onset of oral squamous cell carcinomas (OSCC) is supported by the observation that oral mucosa has similar histological features and properties as vaginal mucosa and that the virus is able to immortalize human keratinocytes in vitro (7).

HR-HPV Types 16 and 18 are the most frequently identified in studies regarding OSCC. Besides other high-risk types, such as 31, 33, 39, 45,5 2,5 8, and 69 (8) and low-risk types (LR-HPV), such as 6 and 11 (5) contributes to the suggestion that HPV has a role in oral cancer.

Multiple HPV types infection within the same lesion may increase the oncogenic potential of the virus and the development of epithelial dysplasia in cervical lesions. However, it is not known whether certain types of HPV

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favor infection by other types of HPV, or whether a superinfection with multiple types of HPV can enhance malignant transformation (8).

In a previous study (9), we evaluate the presence of high-risk type human papillomavirus (16, -18, -31 and -45) in a large, well-defined sample of squamous-cell carcinoma by means of quantitative real-time polymerase chain reaction

The prevalence of HR-HPV in the tumor samples was less than 2% (CI 0.6-3). Matched pairs case-control analysis indicated that the prevalence among controls did not significantly differ with respect to cases and thus did not support a major role of HPV in the etiology of OSCC (9).

Here we test the possible presence of multiple HPV types (low and high risk) in a sample of squamous cell carcinoma limited to oral cavity using the consensus primer Gp, from the HPV L1 gene (10), and Cp, from the E1 gene (11), of the HPV genome, designed to detect a wide range of mucosal HPV types.

MATERIALS AND METHODS

Tissue Collection

Patients were diagnosed between 1990 and 2007, at the Anatomical Pathology Unit, Polytechnic University of the Marche Region, Ancona, Italy. The sample was composed of 215 squamous-cell carcinoma limited to the oral cavity in its strictest definition—ie no tonsil, pharynx or larynx cancers were included—and matched control pairs. In addition, 63 paraffin-embedded tumors without matched controls were analyzed. Tumor samples included at least 80% neoplastic cells and control tissues were obtained from the same patient and anatomical region.

DNA extraction

DNA from paraffin-embedded samples was extracted from two 120 μm thick sections. Slices were placed in xylol for 1h and centrifuged at 12000 g. DNA extraction and purification was performed using the NucleoSpin Tissue Kit (Macherey-Nagel), which can recover DNA fragments as short as 200 bp. The tissue was washed with absolute ethanol twice then incubated overnight at 56°C in 200 μl of lysis solution with 1 mg/ml of proteinase K until it was completely dissolved. Proteinase K was inactivated by heating it to 70°C for 10 min. Cellular debris was removed by spinning at 12000 g for 5 min. The DNA was then purified with NucleoSpin Tissue columns.

Primers and Probe

HPV types were detected using the consensus primers Cp (CPI: 5'-TTATCWTATGCCCAYTGTACCAT-3', CPIIG: 5'-ATGTTAATWSAGCCWCCAAAATT-3') (10) and Gp (GP5+: 5'-TTTGTTACTGTGGTAGATACTAC-3', GP6+: 5'-GAAAAAATAAACTGTAAATCATATTC-3') (11).

The presence and integrity of the DNA in all clinical samples was tested by amplifying the human hydroxymethylbilane synthase isoform 2 (HMBS) gene. The primers and probe were designed using the Primer Express software (Applied Biosystems) (HMBSf: 5'-AAGACACGTTCCACTTTTGATTCA-3'; HMBSr: 5'-ACACAAAAAGAAGGCGCACTTC-3'; HMBSProbe: 5'-AAGCCTCCGAACTGCACACAAACGT C-3'). The probe was quenched with BHQ1 at the 3' end and labeled with JOE at the 5' end.

Real-TimePolymeraseChainReaction

Three real-time polymerase chain reaction runs were performed for each sample. The first reaction detected the human single copy gene HMBS. The other two reactions detected the presence of HPV types using the consensus primer Gp and Cp.

The assays were performed using the Applied Biosystems 7500 Sequence Detection System. HMBS reaction was performed in 20 μl containing 10 μl of 2x TaqMan Universal polymerase chain reaction master mix (Applied Biosystems), 4 μl of DNA purified from samples and 200 nM of each primer and probe. The amplification profile was initiated by a 10 min incubation period at 95°C, followed by a two-step amplification of 15 s at 95°C and 60 s at 57°C for 40 cycles.

Gp and Cp reactions were performed in 20 μl containing 10 μl of 2x SYBR® Green PCR Master Mix (Applied Biosystems), 4 μl of DNA samples and 200 nM of each primer. The RT-PCR program for Cp primers consist of 95 °C for 5 min (95°C for 15 s, 55°C for 60 s, 72°C for 60 s) for 50 cycles, followed by a dissociation step (95°C for 15 s, 60°C for 60 s, 95°C for 15 s). The Gp amplification profile was 95 °C for 5 min (95°C for 15 s, 48°C for 60 s, 72°C for 60 s) for 50 cycles, followed by a dissociation step (95°C for 15 s, 60°C for 60 s, 95°C for 15 s).

PCR products sequencing

HPV type-specific sequences were detected by direct sequencing of positive samples. Amplimers were checked for quality in agarose gel and sent for purification and bidirectional DNA sequencing service to Macrogen (Seul, Korea).

Statistic analyses

Descriptive statistics was performed using Excel spreadsheets (Microsoft Office 2003). Exact Mid-P testing for proportion and for matched pair case-control analysis was performed online at the Open-Epi web site (www.openepi.com).

RESULTS

HPV was detected in 11 tumours out of the 278 examined and only in 1 control (Table I). The overall prevalence rate for HPV was 4.0% (C.I. 2.1-6.8). The matched case-controls analysis (n = 215) demonstrated that the HPV did correlate with squamous cell carcinoma (P value = 0.04); the conditional maximum likelihood estimate of Odds ratio = 7 (C.I. 1.1-159). Among unmatched cases and controls, 3 out of 63 squamous cell carcinoma were HPV positive (P value = 0.43).

Sequencing revealed that 5 samples were positive for HPV type 16, 5 for HPV type 11, and 1 for HPV type 6. The presence of HPV 16 in the tumor sample was

Table I. *Description of cases and control matched pairs, positive for HPV*

T	N	Site	HPV Type	Tumor	Matched Control
2	+	Floor	HPV 16	positive	negative
1	0	Cheek	HPV 16	positive	negative
1	0	Cheek	HPV 16	positive	not avaible
1	+	Cheek	HPV 16	positive	positive
2	+	Tongue	HPV 16	positive	negative
1	0	Tongue	HPV 11	positive	negative
1	0	Tongue	HPV 11	positive	not avaible
2	0	Floor	HPV 11	positive	negative
1	0	Cheek	HPV 11	positive	negative
4	0	Tongue	HPV 11	positive	negative
2	0	Cheek	HPV 6	positive	not avaible

already demonstrated in a previous study (9). HPV 11 was detected in 5 tumours out of the 278 examined. The prevalence rate for HPV 11 was 1.8% (C.I. 0.7-3.9). The matched case-controls analysis (n = 215) demonstrated that HPV 11 alone did not correlate with squamous cell carcinoma (P value = 0.06); the conditional maximum likelihood estimate of Odds ratio = 8 (C.I. 0.4-151).

DISCUSSION

It is widely known that tobacco and alcohol consumption are the major causes of the development of oral squamous cell carcinoma (OSCC) (2). The fact that 15- 20% of patients develop OSCC in the absence of exposure to these agents, however, strongly suggests the existence of other risk factors in oral carcinogenesis, such as the presence of infectious agents (2).

More than 100 different HPV types have been identified and classified as HR (e.g. 16, 18, 31) or LR (e.g. 11, 42, 36) based on their association with cervical carcinoma (12).

HPV can transform oral keratinocytes, especially with chemical carcinogens, but only the high-risk HPV types are able to immortalize these cells.

The carcinogenic role of HR-HPV is due to its oncoproteins HPV-E6 which promotes degradation of the p53 tumor suppressor gene product and HPV-E7 which modifies the pRb tumor suppressor gene product (13).

In this study, we tested for the presence of multiple HPV types (LR and HR) in a large sample of squamous cell carcinomas limited to oral cavity proper and matched controls, using the consensus primer Gp from the HPV L1 (10) gene and the Cp from the E1 gene of the HPV genome (11).

HPV was detected in 11 tumours out of the 278 examined and only in 1 control.

Sequencing revealed that 5 samples were positive for HPV type 16, 5 for HPV type 11, and 1 for HPV type 6.

The presence of HPV 16 in the tumor sample was already demonstrated in a previous study (9) and confirmed here by sequences. However, the results obtained indicated that the prevalence of HPV 16 among controls did not significantly differ with respect to cases excluding a major role of this HPV type in the etiology of OSCC.

At the same way, HPV 11 alone did not correlate with OSCC, as demonstrated in the present study (P value = 0.06; conditional maximum likelihood estimate of Odds ratio = 8 (C.I. 0.4-151)).

HPV 11 and 6 are considered LR- HPV types and are the two most frequent HPV types associated with benign papillomatous lesions of the oral mucosa (6, 14, 15).

Nevertheless, these HPV types are found occasionally in premalignant lesion, such as verrucous carcinoma of the vagina (16), cervical carcinoma and bladder carcinoma (17), in a primary carcinoma of the urethra (18), a case of lung carcinoma (19) as well as a tonsillar carcinoma (20).

Syrjänen et al. analyzing for the presence of HPV DNA in 40 oral carcinomas, disclosed HPV 11, 16 and 18 DNA sequences in 30% of the samples (21). In another series, HPV 6, 11, 16 and 18 DNA were found in 6/51 oral SCCs and in 6/21 oral precancer lesions (22). Löning et al. (23) confirmed this report, detecting HPV 11 and 16 DNA in 3/6 oral carcinomas.

Although many authors show the presence of low-risk HPV in malignant lesion, the present study suggest that there is not relationship between the presence of HPV 6

and 11 types and malignant lesions of the oral cavity in its strictly definition. These results are in accordance with the majority of studies that indicate LR- HPV type associated mainly with benign lesion of this district (6, 14).

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REFERENCES

1. Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. Int J Cancer 1999; 80:827-41.
2. Charfi L, Jouffroy T, de Cremoux P, Le Peltier N, Thioux M, Freneaux P, Point D, Girod A, Rodriguez J, Sastre-Garau X. Two types of squamous cell carcinoma of the palatine tonsil characterized by distinct etiology, molecular features and outcome. Cancer Lett 2008; 260:72-8.
3. Syrjanen S. Human papillomavirus infections and oral tumors. Med Microbiol Immunol 2003; 192:123-8.
4. Campisi G, Panzarella V, Giuliani M, Lajolo C, Di Fede O, Falaschini S, Di Liberto C, Scully C, Lo Muzio L. Human papillomavirus: its identity and controversial role in oral oncogenesis, premalignant and malignant lesions (review). Int J Oncol 2007; 30:813-23.
5. Miller CS, White DK. Human papillomavirus expression in oral mucosa, premalignant conditions, and squamous cell carcinoma: a retrospective review of the literature. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1996; 82:57-68.
6. Campisi G, Giovannelli L, Arico P, Lama A, Di Liberto C, Ammatuna P, D'Angelo M. HPV DNA in clinically different variants of oral leukoplakia and lichen planus. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004; 98:705-11.
7. Shin KH, Min BM, Cherrick HM, Park NH. Combined effects of human papillomavirus-18 and N-methyl-N'-nitro-N-nitrosoguanidine on the transformation of normal human oral keratinocytes. Mol Carcinog 1994; 9:76-86.
8. Hubbard RA. Human papillomavirus testing methods. Arch Pathol Lab Med 2003; 127:940-5.
9. Scapoli L, Palmieri A, Rubini C, Martinelli M, Spinelli G, Ionna F, Carinci F. Low prevalence of human papillomavirus in squamous-cell carcinoma limited to oral cavity proper. Mod Pathol 2009; 22:366-72.
10. de Roda Husman AM, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders PJ. The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol 1995; 76 (Pt 4):1057-62.
11. Tieben LM, ter Schegget J, Minnaar RP, Bouwes Bavinck JN, Berkhout RJ, Vermeer BJ, Jebbink MF, Smits HL. Detection of cutaneous and genital HPV types in clinical samples by PCR using consensus primers. J Virol Methods 1993; 42:265-79.
12. Pillai MR, Nair MK. Development of a condemned mucosa syndrome and pathogenesis of human papillomavirus-associated upper aerodigestive tract and uterine cervical tumors. Exp Mol Pathol 2000; 69:233-41.
13. Scully C. Oral squamous cell carcinoma; from an hypothesis about a virus, to concern about possible sexual transmission. Oral Oncol 2002; 38:227-34.
14. Giovannelli L, Campisi G, Lama A, Giambalvo O, Osborn J, Margiotta V, Ammatuna P. Human papillomavirus DNA in oral mucosal lesions. J Infect Dis 2002; 185:833-6.
15. Burd EM. Human papillomavirus and cervical cancer. Clin Microbiol Rev 2003; 16:1-17.
16. Okagaki T, Clark BA, Zachow KR, Twiggs LB, Ostrow RS, Pass F, Faras AJ. Presence of human papillomavirus in verrucous carcinoma (Ackerman) of the vagina. Immunocytochemical, ultrastructural, and DNA hybridization studies. Arch Pathol Lab Med 1984; 108: 567-70.
17. Oft M, Bohm S, Wilczynski SP, Iftner T. Expression of the different viral mRNAs of human papilloma virus 6 in a squamous-cell carcinoma of the bladder and the cervix. Int J Cancer 1993; 53:924-31.
18. Grussendorf-Conen EI, Deutz FJ, de Villiers EM. Detection of human papillomavirus-6 in primary carcinoma of the urethra in men. Cancer 1987; 60:1832-5.
19. DiLorenzo TP, Tamsen A, Abramson AL, Steinberg BM. Human papillomavirus type 6a DNA in the lung carcinoma of a patient with recurrent laryngeal papillomatosis is characterized by a partial duplication. J Gen Virol 1992; 73 (Pt 2):423-8.
20. Bercovich JA, Centeno CR, Aguilar OG, Grinstein S, Kahn T. Presence and integration of human papillomavirus type 6 in a tonsillar carcinoma. J Gen Virol 1991; 72 (Pt 10):2569-72.
21. Chang F, Syrjanen S, Nuutinen J, Karja J, Syrjanen K. Detection of human papillomavirus (HPV) DNA in oral squamous cell carcinomas by in situ hybridization and polymerase chain reaction. Arch Dermatol Res 1990; 282: 493-7.

22. Syrjanen SM, Syrjanen KJ, Happonen RP. Human papillomavirus (HPV) DNA sequences in oral precancerous lesions and squamous cell carcinoma demonstrated by in situ hybridization. J Oral Pathol 1988; 17:273-8.

23. Loning T, Ikenberg H, Becker J, Gissmann L, Hoepfer I, zur Hausen H. Analysis of oral papillomas, leukoplakias, and invasive carcinomas for human papillomavirus type related DNA. J Invest Dermatol 1985; 84:417-20.