HUMAN PAPILLOMAVIRUS DNA IN ORAL SQUAMOUS CELL CARCINOMAS AND NORMAL ORAL MUCOSA

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Summary. – To elucidate the putative etiologic role of human papillomaviruses (HPV) in oral carcinogenesis, a comparative study was carried out on 62 tissue specimens of oral squamous cell carcinoma (OSCC) and on 62 specimens of histologically normal oral mucosa obtained from the individuals who matched the subjects with OSCC in age, gender, localization of obtained tissue specimens, drinking and smoking habits. Internal control amplification showed that amplifiable DNA was recovered from 59/62 and 61/62 tissue samples of OSCC and normal oral mucosa, respectively. The amplification with two different HPV L1 and one HPV E6 consensus primer sets showed the presence of the HPV DNA genotypes 16, 33, 58 in 5/59 (8.4%) OSCC specimens and HPV genotypes 11, 16, 31, 68 in 4/61 (6.6%) tissue samples of normal oral mucosa tested. In the study in which a comparative examination of the presence of HPV DNA was for the first time performed on the tissue samples of the patients with OSCC and the age- and gender-matched control subjects there was no significant difference in the prevalence of HPV DNA among both study groups. Our results suggest that occasional findings of HPV DNA in OSCC tissue specimens may be the result of an incidental HPV colonization of oral mucosa, rather than of viral infection, and that HPVs play a limited role in the etiopathogenesis of the majority of OSCC.

Key words: human papillomaviruses; oral carcinoma; normal mucosa; Slovenia

Introduction

Squamous cell carcinomas of the oral cavity and pharynx, after the exclusion of the nasopharynx, account worldwide for approximately 220,000 new cases per year in men (5% of all cancers) and 90,000 in women (2% of all cancers) (Parkin *et al.*, 1999; Franceschi *et al.*, 2000a). In some parts of India and South East Asia, OSCC is the most common malignancy accounting for up to 50% of malignant tumors in these regions (Parkin *et al.*, 1999).

Despite extensive research, the etiology and pathogenesis of OSCC remains inconclusive (Franceschi *et al.*, 2000; Scully, 2002). Heavy alcohol and cigarette consumption, especially in combination, as well as betel quid use have been most frequently considered as risk factors for OSCC (Sankaranarayanan *et al*., 1989; Franceschi *et al.*, 2000a). However, only a small proportion of exposed individuals have developed OSCC (Sankaranarayanan *et al*., 1989; Balaram *et al.,* 1995). Moreover, there is an emerging population of OSCC patients who lack exposure to the mentioned risk-factors (Balaram *et al.,* 1995; Scully, 2002). These findings suggest that other factors or agents may play a role in oral carcinogenesis.

Recent evidence suggests that HPV infection can contribute etiologically to some head and neck neoplasms including the subgroup of OSCC (reviewed in Miller and White 1996; Steinberg and DiLorenzo, 1996; Sugerman and Shillitoe, 1997; McKaig *et al.,* 1998; Miller *et al.,* 2001; Scully, 2002). However, the understanding of etiological role of HPV in oral carcinogenesis is hampered by the fact that there is a marked variation in the HPV DNA prevalence rate in tissue specimens of OSCC, varying from 0% (Matzow *et al.,* 1998) to 100% (Watts *et al.,* 1991), as well as in the

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Abbreviations: HPV = human papillomaviruses; PCR = polymerase chain reaction; OSCC = oral squamous cell carcinoma

specimens of normal oral mucosa in which the HPV prevalence rate varies from 0% (Eike *et al.,* 1995; Bouda *et al.,* 2000) to 58.8% (Rice *et al.,* 2000).

To elucidate further the putative etiologic role of HPV in oral carcinogenesis a comparative study of the presence of HPV DNA was carried out on 62 tissue specimens of OSCC and on 62 tissue specimens of histologically normal oral mucosa obtained from the individuals who matched the subjects with OSCC in age, gender, localization of obtained tissue specimens and drinking and smoking habits. The presence of HPV DNA was examined in all tissue specimens using three different polymerase chain reaction (PCR) protocols. To the best of our knowledge, the present study represents the fifth study in which the HPV DNA prevalence was comparatively studied in the patients with OSCC and the age- and gender-matched control subjects and the first study in which a comparative examination was made on tissue samples in both study groups. Additionally, to the best of our knowledge, this study represents the first one in which more than two different PCR protocols were used for the determination of the presence of HPV DNA in either OSCC or normal oral mucosa.

Materials and Methods

OSCC specimens. The first group consisted of 62 OSCC tissue specimens obtained from 62 patients (55 men and 7 women), treated at the Department of Maxillofacial and Oral Surgery, University Medical Center, Ljubljana, Slovenia, from January 1, 1994 to December 31, 1998. The patient's age range was 44–78 years (mean age 58.2). Clinico-pathological data for all the 62 patients are summarized in Table 1. Immediately after resection, the tissue was fixed in 10% neutral-buffered formalin, embedded in paraffin or paraplast, processed to 5 μm-thick sections, and stained with hematoxylin-eosin.

Histologically normal oral mucosa specimens. The second group (controls) consisted of 62 histologically normal oral mucosa tissue specimens obtained by autopsy from the individuals, who matched the case subjects in age (± 5 years), gender, localization of obtained tissue specimens and drinking and smoking habits. Among 62 tissue specimens of normal oral mucosa, 23 were obtained from tongue, 19 from floor of the mouth, 12 from retromolar trigonum, 4 from buccal mucosa and 4 from oropharynx. The autopsy tissue specimens were retrieved from histological files of the Institute of Pathology, Medical Faculty, University of Ljubljana. Immediately after resection, the tissue was fixed in 10% neutral-buffered formalin, embedded in paraffin or paraplast, processed to 5 μm-thick sections, and stained with hematoxylin-eosin.

Isolation of DNA and internal control amplification. DNA was isolated from formalin-fixed paraffin-embedded tissue sections as described previously (Poljak and Cerar, 1993; Poljak *et al.,* 2000). The quality of each DNA sample was verified by the amplification of a 536 bp fragment of ubiquitous human beta-globin gene on real-time PCR apparatus LightCycler® (Roche Applied Science, Mannheim, Germany) using the LightCycler-FastStart DNA Master SYBR Green I Kit and the KM29/RS42 primers as described earlier (Poljak *et al.*, 2002). Successful amplification of the beta-globin gene fragment indicated that the DNA sample was adequate for HPV DNA analysis and that no PCR inhibitors were present.

Detection of HPV DNA. The PCR amplification targeting the portions of the HPV L1 and E6 regions was performed on all samples using the PCR Core Kit^{plus} (Roche Applied Science, Mannheim, Germany) and three different consensus HPV primer sets: PGMY09 and PGMY11 (yielding approximately 450 bp fragments), GP5+ and GP6+ (yielding approximately 140–150 bp fragments), and WD72, WD76, WD66, WD67 and WD154 (yielding approximately 240 bp fragments) as described earlier (Gale *et al.,* 1994; Poljak *et al.,* 1995, 1997; Gravitt *et al.,* 2000). All known precautions to avoid a PCR product carry-over and sample-to-sample contamination were rigorously taken. Different steps of the PCR procedure were performed in separate rooms with different pipettes and with aerosol-resistant tips. Additionally, the uracil-N-glycosylase procedure (included in the PCR Core Kit^{plus}) was used for prevention of false-positive results due to the amplicon carry-over (Longo *et al.*, 1990). To determine HPV genotypes, the PCR products generated from PGMY09 and PGMY11 primers were digested using 7 restriction endonucleases (*Bam*HI*, Dde*I*, Hae*III*, Hinf*I*, Pst*I*, Rsa*I, and *Sau*3AI) and analyzed by agarose gel electrophoresis as described earlier (Poljak and Cerar, 1993; Bernard *et al.*, 1994; Poljak *et al.,* 1998a). A DNA 50 bp ladder (Roche Applied Science, Mannheim, Germany) was used as size marker.

Statistical analysis was performed by Student's test and χ^2-test.

Results

The internal control amplification with human beta-globin primers KM29/RS42 showed that the amplifiable DNA was recovered from 59 of 62 and 61 of 62 tissue specimens of OSCC and normal oral mucosa, respectively. Four tissue specimens which failed in the internal control amplification were excluded from further analysis (patients No. 9, 25, and 47 in Table 1) and one tissue specimen of normal mucosa obtained from the mouth floor.

The amplification with two different HPV L1 and one HPV E6 consensus primer sets showed the presence of HPV DNA in 5 of 59 (8.4%) OSCC specimens tested (i.e. in two cases of oropharyngeal OSCC, in two cases of OSCC of tongue, and in one retromolar OSCC (Table 1). All three consensus primer sets recognized HPV DNA in 4 of 5 HPV DNA-positive OSCC specimens (Fig. 1, lanes 2, 3, 5, 6). In one OSCC specimen HPV DNA was recognized only by the PGMY and WD consensus primer sets (Fig. 1, lane 4). For determining HPV genotypes, PGMY09/PGMY11 PCR products were digested with 7 restriction endonucleases and analyzed by agarose gel electrophoresis. The restriction patterns were found to be unique for the HPV genotype 16 in both cases of oropharyngeal OSCC and in one case of OSCC of the tongue, for the HPV genotype 33 in retromolar

Table 1. Clinicopathological data and HPV infection characteristics obtained in 62 Slovenian patients with OSCC

Patient No.	Sex	Age	Site	Grade	Stage	HPV status
1	m	44	tongue	II	T2N0	–
2	f	60	mouth floor	II	T2N0	–
3	m	52	mouth floor	II	T4N2b	–
4	f	44	retromolar trigonum	III	T4N2b	–
5	f	71	mouth floor	II	T4N0	–
6	m	45	retromolar trigonum	I	T4N0	–
7	m	78	tongue	II	T2N0	–
8	m	62	floor of mouth	II	T1N0	–
9	m	63	floor of mouth	II	T3N0	ND
10	m	55	oropharynx	II	T1N0	–
11	m	69	floor of mouth	I	T3N0	–
12	m	51	floor of mouth	III	T4N0	–
13	m	70	floor of mouth	II	T4N0	–
14	m	41	retromolar trigonum	II	T4N0	–
15	m	54	tongue	II	T2N0	–
16	m	55	floor of mouth	II	T2N0	–
17	m	72	floor of mouth	II	T2N1	–
18	m	66	retromolar trigonum	III	T2N0	–
19	m	71	oropharynx	I	T2N0	HPV 16
20	m	63	tongue	II	T3N3b	
21	m	46	tongue	II	T2N1	–
22	m	61	tongue	III	T4N2	–
23	m	48	oropharynx	II	T2N0	–
24	m	60	tongue	II	T2N0	–
25	m	56	retromolar trigonum	III	T3N0	ND
26	m	46	retromolar trigonum	II	T4N0	–
27	m	67	tongue	III	T4N0	–
28	m	41	tongue	II	T2N1	–
29	m	68	floor of mouth	III	T4N3b	–
30	m	64	retromolar trigonum	II	T2N1	HPV 33
31	m	42	tongue	I	T2N0	–
32	f	50	tongue	II	T2N0	HPV 16
33	m	58	tongue	II	T4N0	–
34	m	61	retromolar trigonum	I	T3N1	
35	m	78	tongue	II	T2N0	–
36	f	51	floor of mouth	II	T2N1	–
37	m	70	tongue	I	T4N0	–
38	m	60	tongue	II	T1N0	–
39	m	58	retromolar trigonum	III	T1N0	–
40	m	63	floor of mouth	II	T2N0	–
41	m	49	tongue	II	T3N0	–
42	m	54	tongue	I	T3N2b	–
43	f	66	floor of mouth	II	T3N0	–
44	m	72	tongue	III	T4N1	–
45	m	54	oropharynx	II	T3N1	HPV 16
46	m	46	floor of mouth	II	T2N0	–
47	m	77	buccal mucosa	II	T2N0	ND
48	m	59	tongue	I	T4N0	–
49	m	46	buccal mucosa	II	T2N1	–
50	m	58	floor of mouth	II	T2N0	–
51	m	71	tongue	I	T4N0	–
52	f	52	floor of mouth	III	T2N0	–
53	m	62	tongue	III	T2N0	–
54	m	55	buccal mucosa	II	T2N0	–
55	m	52	buccal mucosa	II	T4N0	–
56	m	64	tongue	II	T3N1	HPV 58
57	m	55	floor of mouth	II	T2N0	–
58	m	66	floor of mouth	I	T2N1	–
59	m	54	retromolar trigonum	II	T4N0	–
60	m	52	retromolar trigonum	III	T1N0	–
61	m	63	tongue	II	T4N2	–
62	m	47	retromolar trigonum	II	T2N0	–

m = male; f = female; ND = not done due to negative internal control amplification results; (–) = negative.

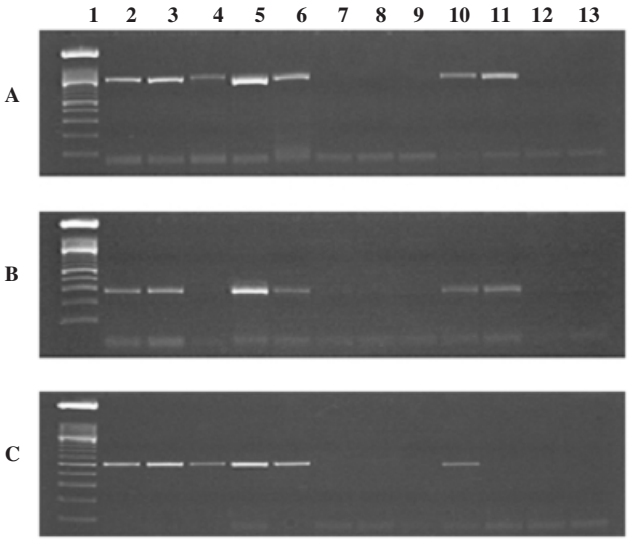

Fig. 1

Representative products of PCR amplification of HPV DNA from OSCC specimens (lanes 2–9) and specimens of normal oral mucosa (lanes 10–13) using primers PGMY09/PGMY11 (part A app. 450 bp fragments), GP5+/GP6+ (part B 140-150 bp fragments), and WD72/WD76/WD66/WD67/WD154 (part C; app. 240 bp fragments)

DNA 50 bp ladder (lane 1); patient No. 19 (lane 2); patient No. 45 (lane 3); patient No. 32 (lane 4); patient No. 30 (lane 5); patient No. 56 (lane 6); patient No. 53 (lane 7); patient No. 54 (lane 8); patient No. 55 (lane 9); HPV 31-positive tissue specimen of normal mucosa from oropharynx (lane 10); HPV 68-positive tissue specimen of normal mucosa from mouth floor (lane 11); HPV-negative tissue specimens normal buccal mucosa and tongue, respectively (lanes 12–13).

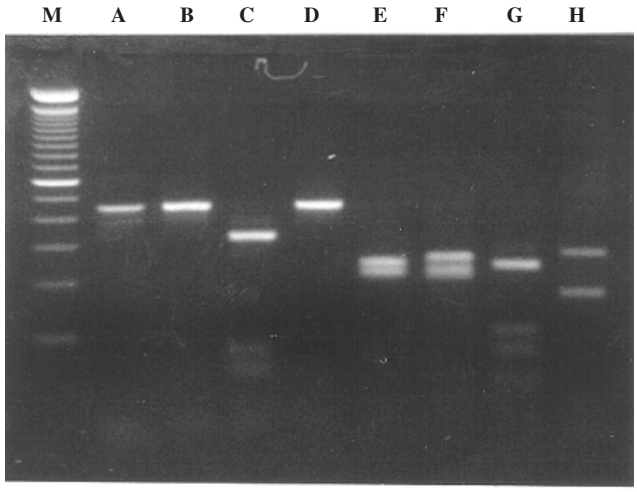

Fig. 2

Determination of HPV genotype by restriction analysis of the PGMY09/PGMY11 PCR product

Agarose gel ekectrophoresis patterns of PCR product of DNA extracted from a case of OSCC (patient No. 30) cleaved with *Bam*HI (lane B; 449 bp), *Dde*I (lane C; 320, 77 and 52 bp), *Hae*III (lane D; 449 bp), *Hinf*I (lane E; 234 and 215 bp), *Pst*I (lane F; 242 and 207 bp), *Rsa*I (lane G; 236, 102, 72 and 39 bp), and *Sau3*AI (lane H; 267, 162 and 20 bp). DNA 123 bp ladder as size marker (Gibco-BRL, lane M); negative control (lane A).

OSCC and for the HPV genotype 58 in one case of OSCC of the tongue (Table 1 and Fig. 2).

In contrast, HPV DNA was detected in 4 of 61 (6.6%) tissue specimens of normal oral mucosa tested. In three tissue specimens of normal oral mucosa, the HPV DNA was detected using all three HPV consensus primer sets and in one tissue specimen with PGMY and GP primer sets only (Fig 1, lane 11). Restriction analysis of the PGMY09/PGMY11 PCR products showed the presence of the HPV genotypes 11 and 16 in two tongue specimens and the HPV genotypes 31 and 68 in tissue specimens of normal mucosa obtained from oropharynx and mouthfloor, respectively (Bernard *et al.*, 1994).

Discussion

In the last decade, using different molecular approaches, the presence of HPV DNA has been detected in squamous cell neoplasms of several organs (reviewed in IARC, 1995; zur Hausen, 1996; Syrjänen and Syrjänen, 2000). However, a wealth of molecular, experimental, epidemiological, morphological and clinical data has established an important role for infection with a subset of HPV genotypes (the so-called "high risk" genotypes) solely in the case of cervical carcinoma. Since the HPV prevalence rate in cervical carcinoma worldwide has already reached 99.7% (Walboomers *et al.,* 1999), persistent HPV infection appears to be an essential, although not sufficient factor for the development of cervical carcinoma. In addition to cervical carcinoma, high risk HPV genotypes are also found frequently in other anogenital tract carcinomas including penile, anal, and vulvar cancers (reviewed by Syrjänen and Syrjänen, 2000), although this association is not so convincing as in the case of cervical carcinomas.

In contrast, for oral squamous cell carcinomas, there is still controversy over the real prevalence of HPV infection and its possible significance as an oncogenic agent (Miller and White 1996; Steinberg and DiLorenzo, 1996; Sugerman and Shillitoe, 1997; McKaig *et al.,* 1998; Miller *et al.,* 2001; Scully, 2002). E.g., early studies using Southern-blot, dot-blot and *in situ* hybridization techniques have found a quite different prevalence of HPVs in OSCC, ranging from 0% to 76.4% (reviewed by Sugerman and Shillitoe, 1997). This wide difference in HPV prevalence rates was even greater in later studies in which a more sensitive PCR was used for detection of HPVs, with the findings ranging from 0% to 100% (reviewed by Miller *et al.,* 2001). A number of factors, mainly

technical, may account for the observed differences in HPV prevalence rates in different studies. PCR is an exquisitely sensitive tool, sensitive to false positive results from a variety of sources. False positives can originate from difficulties in interpretation of results or more likely from true HPV-negative specimens contaminated with HPV DNA during collection of the samples, sectioning of paraffin-embedded tissue blocks, DNA extraction or as a result of the carry-over of the amplified DNA from the specimens processed previously. Although some of the classical nonspecific precautions to minimize the chance of PCR contamination were more or less taken in almost all PCR-based studies on HPV prevalence in OSCC, to the best of our knowledge, in a single study specific amplicon-inactivation methods like a post-PCR cross-linkage method with isopsoralen, pre-PCR uracil N-glycosylase method or post-PCR alkaline hydrolysis have been used. Therefore we are convinced that high HPV prevalence rates obtained in some studies, mainly in those performed in early 1990s, were influenced by some false positives. These false positive results led to overestimated prevalence rates of HPVs in normal, benign and malignant tissues of oral mucosa and, consequently, initiated a number of debates and confusions regarding the etiologic role of HPV infection in the development of OSCC.

To overcome possible PCR contamination problems, which in our opinion cannot be successfully eliminated solely by classical anticontamination procedures, we used an uracil-N-glycosylase method for prevention of false-positive results in our study. This method incorporated into the PCR Core Kit^{plus} has been proven capable of eliminating efficiently the amplicon carry-over in a number of studies (reviewed in Poljak *et al.,* 1998b) and has developed in an indispensable part of all commercially available PCR kits since 1996 (e.g. the Roche Amplicor assays). Additionally, to minimize the possibility of false-negative results due to the heterogeneity of HPV genotypes, three different PCR consensus primers targeting two different HPV genes were used for determination of the presence of HPV DNA in either OSCC or normal oral mucosa. Using such an assay protocol, designed to detect a maximum number of HPV genotypes with minimum possibility of false-positive results, we were able to detect HPV DNA in 8.4% of OSCC specimens, which is among the lowest HPV DNA prevalence rates recognized in OSCC series tested to date. However, in our opinion, to elucidate the real clinical meaning of the established prevalence of HPV DNA among OSCC specimens, it is indispensable to detect a "background" prevalence of HPV DNA in the oral mucosa which, in fact, represents the HPV prevalence in normal oral mucosa in the age- and gender-matched control subjects. To the best of our knowledge, only four studies to date have made a comparative investigation of the HPV DNA prevalence in the patients with OSCC and the age- and gender- matched control subjects (Maden *et al.,* 1992; Schwartz *et al.,* 1998; Smith *et al.,* 1998; Summersgill *et al.,* 2000). Although in all the four studies the significantly higher prevalence of HPV DNA in the patients with OSCC compared with control subjects has been recognized, it should be stressed that these studies have compared HPV prevalence rates determined in different types of clinical specimens obtained from the patients with OSCC and controls. Namely, the HPV DNA prevalence has been studied in tissue specimens of OSCC versus oral rinses or exfoliated oral cells obtained by soft-bristled toothbrush from control subjects. Therefore, one could argue that the obtained differences in the HPV DNA prevalence rates between the patients with OSCC and controls could be a result of the usage of different types of clinical specimens. To overcome this problem, we used the same type of clinical specimens e.g. formalin-fixed paraffin-embedded tissue specimens for determination of the HPV DNA prevalence among the patients with OSCC and controls. With such a study design, we were unable to determine any significant difference in the prevalence of HPV DNA between the patients with OSCC and the age- and gender-matched control subjects (8.4% vs. 6.6%; $p = 0.96$). Interestingly, similar results have been also obtained by Schwartz *et al.* (1998), in which they have investigated the HPV DNA prevalence in exfoliated oral cavity cells obtained from both the patients with OSCC and the control subjects. Namely, although Schwartz *et al.* (1998) have obtained a significantly different prevalence of HPV DNA in tumor tissue specimens versus exfoliated oral cavity cells (25.8% vs. 9.2%, respectively) from both the patients with OSCC and the control subjects. On the contrary, the prevalence of HPV DNA was almost the same (9.3% vs. 9.2%, respectively) when they compared the same type of clinical specimens, e.g. exfoliated oral cavity cells obtained from both the groups.

In conclusion, in the study in which a comparative examination of the presence of HPV DNA was made for the first time on tissue specimens from the patients with OSCC and the age- and gender-matched control subjects, we found similar prevalence of HPV DNA in both groups under comparison. Our results suggest that occasional findings of HPV DNA in OSCC tissue specimens may result not from viral infection, but rather from an incidental HPV colonization of oral mucosa. According to our results, at least in this part of Europe, it seems that HPVs play a limited role in the etiopathogenesis of the majority of OSCC.

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