"High Risk" HPV Types Are Frequently Detected in Potentially Malignant and Malignant Oral Lesions, But Not in Normal Oral Mucosa

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Studies on the involvement of the human papillomavirus (HPV) in initiation and progression of oral neoplasia have generated conflicting results. The observed discrepancy is attributable mainly to the varying sensitivity of the applied methodologies and to epidemiologic factors of the examined patient groups. To evaluate the role of HPV in oral carcinogenesis, we analyzed 53 potentially neoplastic and neoplastic oral lesions consisting of 29 cases of hyperplasia, 5 cases of dysplasia, and 19 cases of squamous cell carcinomas, as well as 16 oral specimens derived from healthy individuals. A highly sensitive nested polymerase chain reaction (PCR) assay was used, along with type-specific PCR, restriction fragment length polymorphism analysis, dot blotting, and nonisotopic *in situ* hybridization. Nested PCR revealed the presence of HPV DNA in 48 of the 53 (91%) pathologic samples analyzed, whereas none (0%) of the normal specimens was found to be infected. Positivity for HPV was independent of histology and the smoking habits of the analyzed group of patients. At least one "high risk" type, such as HPV 16, 18, and 33, was detected by type-specific PCR in 47 (98%) infected specimens, whereas only 1 (2%) squamous cell carcinoma was solely infected by a "low risk" type (HPV 6). HPV 16 was the prevailing viral type, being present in 71% of infected cases. Single HPV 16 and HPV 18 infections were confirmed by restriction fragment length polymorphism. HPV 58 was detected by dot blotting in three hyperplastic lesions. HPV positivity and genotyping

were further confirmed, and the physical status of this virus was evaluated by nonisotopic *in situ* hybridization. Diffuse and punctate signals, indicative of the episomal and integrative pattern of HPV infection, were observed for low- and high-risk types, respectively. Our findings are suggestive of an early involvement of high-risk HPV types in oral carcinogenesis.

KEY WORDS: Human papillomavirus, Oral carcinoma, Oral dysplasia, Oral mucosa.

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Cancer of the oral cavity is the sixth most common malignancy in developed countries, representing almost 3% of malignant tumors (1). In India and other regions of southeast Asia, it is the predominant malignancy, accounting for up to 50% of all cancers (2). This high incidence of oral cancer is attributable mainly to the habit of tobacco chewing and, secondly, to alcohol and betel quid consumption (3). However, that a large part of the population develops oral cancer without exposure to these risk factors suggests that additional causes, such as genetic predisposition, diet, and viral agents, may participate in oral carcinogenesis (4). Viruses that have been implicated in oral neoplasias are Epstein-Barr virus, human herpesvirus 8, human cytomegalovirus, and, particularly, human papillomaviruses (HPVs) (5, 6).

HPV is a small, epitheliotropic, nonenveloped DNA virus. The HPV genome consists of 7200 to 8000 base pairs of closed-circular double-stranded DNA, containing up to 10 open reading frames. HPV belongs to a large family of viruses, the papovaviridae, and thus far, more than a 100 different types have been identified in humans. Some of these types, such as 16, 18, 33, and 58, seem to play

a role in the development of certain human tumors and are characterized as "high risk" types (7). Squamous cell malignant lesions of the cervix are infected mainly by high-risk viral types (8), whereas other organs, such as the larynx, are infected by a wide range of HPV types with varying tumorigenic potential (7, 9).

The presence of HPV genome in oral mucosa has been reported with various percentages and genotyping patterns (Table 1). Furthermore, most of the existing reports have selectively examined certain proliferative conditions of the oral cavity. To gain further insight on the involvement of the virus in carcinogenesis of the oral cavity, we comprehensively investigated HPV infection in 53 oral lesions, representing the whole spectrum of potentially neoplastic and neoplastic stages. The presence of HPV was also examined in 16 oral specimens derived from a group of healthy individuals. The experimental procedure consisted of a combination of techniques with the highest levels of sensitivity and specificity available. To our knowledge, this is the first study to examine HPV infection in oral hyperplasias, dysplasias, and squamous cell carcinomas (SqCCs), as well as in normal oral mucosa, by nested polymerase chain reaction (NPCR), type-specific PCR (TS-PCR), restriction fragment length polymorphism (RFLP) analysis, dot blotting (DB), and nonisotopic *in situ* hybridization (NISH).

MATERIALS AND METHODS

Samples

Preneoplastic and neoplastic oral specimens

Fifty-three archival, formalin-fixed, paraffin-embedded oral specimens consisting of 29 hyperplasias, 5 dysplasias, and 19 SqCCs were obtained from the routine files of the Department of Dermatology, Syngros Hospital, Athens, Greece. All samples were biopsies and surgical specimens derived from the cheeks (22 cases), tongue (13 cases), alveolar mucosa (7 cases), gingiva (5 cases), lips (3 cases), palate (1 case), modiolus (1 case), and floor of the mouth (1 case). Thirty-one specimens were derived from male patients, and 22 were derived from female patients. The age of patients ranged from 27 to 84 years, with a mean age of 52.7 years. Thirty-eight samples (71.7%) were derived from heavy smokers (>20 cigarettes per day), and the remaining 15 samples (28.3%) were derived from nonsmokers. Cancerous lesions were classified into poorly differentiated (one case), moderately differentiated (seven cases), well-differentiated (seven cases), and *in situ* carcinomas (three cases), according to Smith and Pindborgs' criteria (46) (Table 2).

Normal oral specimens

Sixteen cheek and tongue cytologic scrapes were obtained from a group of healthy subjects, consisting of 6 men and 10 women. Ages ranged from 24 to 65 years, with a mean age of 48.3 years. Five specimens (31.3%) were derived from heavy smokers, and 11 (68.7%) were derived from nonsmokers (Table 2).

Experimental Procedure

HPV genomic DNA was detected with a highly sensitive NPCR assay. To avoid false-negative results due to low DNA yield or low number of viral copies, extracted DNA integrity was confirmed by amplifying a 523-bp fragment of *p53* exons 5–6, which is larger than the HPV fragments obtained by the PCR (9). In the first step of the NPCR assay, we used the degenerate L1 consensus primers MY09 and MY11 (47), which provide a sensitivity comparable to that of Southern blotting (1 viral copy per 10² cells) (48). The second step of the assay was performed using the nested consensus primers GP5+/GP6+, which detect a broader spectrum of viral types than the "first-generation" GP5/GP6 primers and present the highest available sensitivity compared with any single technique (1 HPV copy per 10⁴ to 10⁵ cells) (49, 50). Positive NPCR samples were analyzed by TS-PCR with primers designed for the detection of HPV types 6, 11, 16, 18, 31, and 33 (47). Samples infected solely by HPV 16 or HPV 18 were further analyzed by RFLP, because specimens with coinfections present an indefinite pattern in RFLP assays (51). Confirmation of NPCR results and detection of HPV 58 were carried out by DB assay (52–54). Finally, the physical status of the virus in infected specimens was investigated by NISH.

DNA extraction

Analysis of preneoplastic and neoplastic specimens was performed on six adjacent 5- μ m sections of each paraffin embedded tissue, with the first section being stained with hematoxylin and eosin to visualize the extent of the abnormal cells. The remaining slices were collected in a sterile 1.5-mL microcentrifuge tube. DNA was extracted by a method generating high-quality and high-quantity DNA from paraffin-embedded tissues, as previously described (55). Normal specimens were initially placed in 2-mL neutral phosphate buffered saline in sterile 5-mL centrifuge tubes. Samples were centrifuged at 2000 rpm for 15 min, and DNA was extracted from the collected cell pellets after the QIAamp DNA Extraction procedure (QIAGEN; Bio-Analytica, Athens, Greece). To avoid cross-contamination at any step of the procedure, paraffin-embedded compounds were cut between

TABLE 1. Summary of Reports on HPV Infection in Oral Mucosa^a

Study	References	Country	Year	Normal Samples	Pathologic Specimens			HPV Positive	Infection by Types ^b										Detection Methods					
					Hyperpl	Dyspl	Oral SqCC		2	6	11	16	18	33	58	Other ^c	SB	PCR	DB	ISH	RFLP	SEQ	IHC	EM
North America																								
Holladay & Gerald	10	USA	1993	6 (17%)	9 (44%)	36 (25%)	39 (18%)	20 (24%)	11	21	1						+							
Woods <i>et al.</i>	11	USA	1993	9 (67%)	-	-	18 (78%)	14 (78%)	4	1	7	2					+							
Brandwein <i>et al.</i>	12	USA	1994	-	-	-	64 (25%)	16 (25%)								+								
Paz <i>et al.</i>	13	USA	1997	-	-	-	92 (20%)	18 (20%)	2	13						3	+							
Portugal <i>et al.</i>	14	USA	1997	-	-	-	58 (7%)	4 (7%)								+								
Wilczynski <i>et al.</i>	15	USA	1998	-	-	-	19 (63%)	12 (63%)		9	1					2	+		+					
South America																								
Miguel <i>et al.</i>	16	Brazil	1998	34 (12%)	-	-	34 (12%)	4 (12%)		4						+		+						
Premoli-De-Percoco <i>et al.</i>	8	Venezuela	1998	-	-	-	50 (70%)	35 (70%)	7	28							+		+					
Africa																								
Van Rensburg <i>et al.</i>	17	South Africa	1995	-	-	-	66 (0%)	0 (0%)										+						+
Asia																								
Chang <i>et al.</i>	18	China	1989	17 (6%)	3 (100%)	-	17 (76%)	16 (80%)		16						+								
Heinzel <i>et al.</i>	19	India	1996	-	-	-	91 (74%)	67 (74%)																
Chatterjee <i>et al.</i>	20	India	1997	-	-	-	39 (69%)	26 (69%)		19						+			+					
D'Costa <i>et al.</i>	3	India	1998	48 (31%)	-	80 (34%)	100 (15%)	42 (23%)		42						+								
Wen <i>et al.</i>	21	Japan	1997	-	15 (33%)	-	45 (33%)	16 (32%)		16	12					+						+		
Mineta <i>et al.</i>	22	Japan	1998	-	-	-	14 (21%)	3 (21%)		3						+								
Shinji <i>et al.</i>	23	Japan	1999	7 (0%)	-	-	15 (0%)	0 (0%)								+								
Koh <i>et al.</i>	24	Korea	1998	-	-	-	42 (52%)	22 (52%)		13	5	4				+								
Europe																								
Cerovac <i>et al.</i>	25	Croatia	1996	-	-	-	25 (20%)	5 (20%)										+						
Eike <i>et al.</i>	26	Denmark	1995	61 (0%)	-	-	-	-								+								+
Syrjanen <i>et al.</i>	27	Finland	1983	-	-	-	40 (40%)	16 (40%)																
Atula <i>et al.</i>	28	Finland	1997	-	-	-	41 (20%)	8 (20%)		4						+								
Fouret <i>et al.</i>	29	France	1995	42 (0%)	-	3 (0%)	21 (8%)	2 (8%)								+								
Gassenmaier & Hornstein	30	Germany	1988	-	202 (10%)	103 (18%)	68 (24%)	56 (15%)	4	6	7	12				+								
Honig	31	Germany	1992	-	-	-	12 (60%)	7 (60%)								+			+					
Ostwald <i>et al.</i>	32	Germany	1994	97 (1%)	-	-	26 (61.5%)	16 (61.5%)								+								
Arndt <i>et al.</i>	33	Germany	1997	-	14 (86%)	1 (100%)	-	13 (87%)	13	3						+		+						
Riethdorf <i>et al.</i>	34	Germany	1997	-	-	-	80 (44%)	35 (44%)								+								
Andl <i>et al.</i>	35	Germany	1998	-	-	-	64 (17%)	11 (17%)		8	1					+								
Lambropoulos <i>et al.</i>	36	Greece	1997	169 (9.5%)	-	-	-	-	7	1	4					+								
Present study		Greece	1999	16 (0%)	29 (86%)	5 (100%)	19 (95%)	48 (90%)	7	1	34	17	6	3		+		+						
Lo Muzio <i>et al.</i>	37	Italy	1997	-	-	21 (48%)	5 (40%)	17 (66%)	3	9								+						
Cruz <i>et al.</i>	38	Netherlands	1996	-	-	-	35 (54%)	19 (54%)	1	15						4	+							
Snijders <i>et al.</i>	39	Netherlands	1996	-	-	-	25 (20%)	5 (20%)		5							+							
Matzow <i>et al.</i>	40	Norway	1998	-	5 (0%)	-	35 (3%)	1 (3%)								+								
Adams <i>et al.</i>	41	Switzerland	1999	4 (50%)	-	-	20 (30%)	6 (30%)		5	1					1	+					+		
Jalal <i>et al.</i>	42	UK	1992	48 (44%)	-	-	-	-		21							+							
Scholes <i>et al.</i>	43	UK	1997	-	-	-	8 (62%)	5 (62%)				5					+							
Elamin <i>et al.</i>	44	UK	1998	-	-	12 (33%)	28 (50%)	18 (45%)	8	10						+								
Penhallow <i>et al.</i>	45	UK	1998	-	3 (67%)	9 (56%)	28 (50%)	18 (53%)	8	10							+							

HPV, human papillomavirus; Hyperpl, hyperplasia; Dyspl, dysplasia; SqCC, squamous cell carcinoma; SB, Southern or slot blot; PCR, polymerase chain reaction; DB, dot blot; ISH, *in situ* hybridization; RFLP, restriction fragment length polymorphism; SEQ, sequencing; IHC, immunohistochemistry; EM, electron microscopy.

^a Studies analyzing fewer than 10 samples were not included in this table.

^b Detected either alone or in combination with other types.

^c Type determination not achieved by applied methodology.

TABLE 2. Summary of Clinicopathologic Data and HPV Infection Results in Hyperplastic, Dysplastic, and Cancerous Lesions and in Normal Specimens

Sample No.	Sex	Age	Smoking	Site	Histology	HPV Infection	Viral Type
Hyperplastic, Dysplastic, and Cancerous Lesions							
1	F	45	+	Alveolar mucosa	Hyperplasia	+	16
2	M	75	—	Alveolar mucosa	Hyperplasia	+	16
3	F	40	+	Alveolar mucosa	Hyperplasia	+	18
4	M	37	+	Alveolar mucosa	Hyperplasia	—	—
5	M	44	+	Cheek	Hyperplasia	+	16
6	M	50	+	Cheek	Hyperplasia	+	16
7	F	57	—	Cheek	Hyperplasia	+	18
8	M	27	+	Cheek	Hyperplasia	+	16
9	M	39	+	Cheek	Hyperplasia	+	33
10	M	52	+	Cheek	Hyperplasia	+	16
11	M	52	+	Cheek	Hyperplasia	+	16
12	F	36	+	Cheek	Hyperplasia	+	6, 16, 58
13	M	53	+	Cheek	Hyperplasia	+	6, 16, 58
14	M	48	+	Cheek	Hyperplasia	+	16
15	M	57	+	Cheek	Hyperplasia	—	—
16	M	41	+	Cheek	Hyperplasia	+	6, 16, 58
17	M	46	+	Cheek	Hyperplasia	+	16
18	M	36	+	Cheek	Hyperplasia	—	—
19	F	45	+	Floor of mouth	Hyperplasia	+	18
20	F	55	—	Gingiva	Hyperplasia	+	16
21	F	47	+	Gingiva	Hyperplasia	+	18, 33
22	M	37	+	Gingiva	Hyperplasia	+	18
23	F	53	+	Gingiva	Hyperplasia	+	18
24	M	40	—	Modiolus	Hyperplasia	+	16
25	F	34	+	Palate	Hyperplasia	—	—
26	F	53	—	Tongue	Hyperplasia	+	6, 16, 18
27	F	55	—	Tongue	Hyperplasia	+	16, 18
28	F	51	+	Tongue	Hyperplasia	+	16
29	M	56	+	Tongue	Hyperplasia	+	6, 16, 33
30	F	45	—	Cheek	Dysplasia	+	18
31	M	63	—	Cheek	Dysplasia	+	6, 11, 16, 18, 31, 33
32	M	62	—	Cheek	Dysplasia	+	16, 18, 33
33	M	64	—	Lip	Dysplasia	+	18
34	F	42	+	Tongue	Dysplasia	+	16
35	F	71	+	Alveolar mucosa	SqCC (MD)	+	16
36	M	65	+	Alveolar mucosa	SqCC (WD)	—	—
37	M	56	+	Alveolar mucosa	SqCC (ISC)	+	18
38	M	46	+	Cheek	SqCC (MD)	+	16
39	F	68	+	Cheek	SqCC (MD)	+	16
40	M	59	—	Cheek	SqCC (MD)	+	16
41	F	67	+	Cheek	SqCC (WD)	+	16
42	M	62	+	Cheek	SqCC (WD)	+	6
43	M	80	+	Gingiva	SqCC (WD)	+	16
44	M	54	+	Lip	SqCC (ISC)	+	16
45	M	64	—	Lip	SqCC (WD)	+	16
46	F	77	—	Tongue	SqCC (WD)	+	16, 18
47	F	50	+	Tongue	SqCC (ISC)	+	18
48	M	29	+	Tongue	SqCC (MD)	+	18
49	M	41	—	Tongue	SqCC (WD)	+	16, 33
50	F	84	—	Tongue	SqCC (PD)	+	16
51	F	57	+	Tongue	SqCC (MD)	+	16
52	F	84	+	Tongue	SqCC (WD)	+	18
53	M	53	+	Tongue	SqCC (MD)	+	16
Normal Specimens							
1	M	24	+	Cheek-tongue	—	—	—
2	M	35	+	Cheek-tongue	—	—	—
3	M	48	—	Cheek-tongue	—	—	—
4	M	49	+	Cheek-tongue	—	—	—
5	M	63	+	Cheek-tongue	—	—	—
6	M	65	+	Cheek-tongue	—	—	—
7	F	37	—	Cheek-tongue	—	—	—
8	F	40	—	Cheek-tongue	—	—	—
9	F	43	—	Cheek-tongue	—	—	—
10	F	44	—	Cheek-tongue	—	—	—
11	F	44	—	Cheek-tongue	—	—	—
12	F	50	—	Cheek-tongue	—	—	—
13	F	52	—	Cheek-tongue	—	—	—
14	F	55	—	Cheek-tongue	—	—	—
15	F	61	—	Cheek-tongue	—	—	—
16	F	63	—	Cheek-tongue	—	—	—

HPV typing results are based on type-specific analysis, except for the specimens infected by HPV 58, which were examined by dot blot assay.
 HPV, human papillomavirus; F, female; M, male; PD, poorly differentiated; MD, moderately differentiated; WD, well differentiated; ISC, *in situ* carcinoma.

samples and were subsequently subjected to DNA extraction and PCR analysis.

HPV-positive and -negative controls

Control samples were selected as previously described (9). Briefly, cell lines CasKi and SiHa served as positive controls for HPV 16, HeLa for HPV 18, positive cervical warts for HPV 6 and 11, and cervical SqCCs for HPV 31, 33, and 58. Cell line K562 was used as negative control (56).

PCR analysis

Primers. Detection of HPV sequences was performed by two sets of consensus primers, MY09/MY11 and GP5+/GP6+, which amplify a 448-bp and an internal 138-bp region, respectively, in the highly conserved L1 HPV gene (47, 50). Positive specimens were analyzed by TS-PCR for HPV types 6, 11, 16, 18, 31, and 33 infection, as previously described (Fig. 1) (9).

PCR. Amplifications were performed in a DNA-free room with DNA-free equipment to minimize the risk of contamination. Furthermore, negative controls of water were included between samples. DNA was transferred to the PCR buffer with aerosol-resistant pipette tips. The thermal profiles used were described in a previous study (9). After amplification, the reaction products were electrophoresed on 2% agarose and visualized by ethidium bromide staining (Fig. 1).

RFLP analysis

Single HPV 16 and HPV 18 infections were confirmed by RFLP using the following procedure: 25 μ L MY09/MY11 PCR products were incubated overnight at 37° C with 0.5 μ L *Hae*III (20 u/mL), 0.5 μ L *Rsa*I (20 u/mL), and 0.5 μ L *Pst*I (20 u/mL) (Biolabs, BioLine, Athens, Greece) in a 0.5-mL microcentrifuge tube. Digestion products were electrophoresed at 110 V for 90 min on 3% MetaPhor agarose (FMC BioProducts, BioAnalytica) and evaluated according to the patterns reported by Lungu *et al.* (Fig. 1) (51).

Dot blotting

Infected specimens, which were identified by the NPCR assay, were further analyzed by DB, using type-specific oligomer probes (52–54, 57). NPCR products were diluted 1:10 and denatured for 10 min at 95° C. One microliter of the denatured DNA was applied on the nylon membrane and placed under ultraviolet light for 3 min, followed by baking at 80° C for 1 to 2 h. The membranes were placed into prehybridization buffer (0.02% sodium dodecyl sulfate, 1 g blocking reagent in 2 $\times$ standard saline citrate) for 1 h at 55° C and hybridized overnight at the same temperature using individual 5'digoxige-

FIGURE 1. Representative human papillomavirus (HPV) nested polymerase chain reaction (NPCR) and type-specific PCR products, as well as restriction fragment length polymorphism digestion products. DNA samples were electrophoresed on 2% agarose and 3% MetaPhor agarose, respectively, and viewed by ethidium bromide staining. **A**, amplification products with consensus HPV primers. M, Φ $\times$ 174/*Hae*III DNA marker; lanes 1 to 2, 523-bp *p53* gene amplified fragment from two specimens; lane 3, first-step NPCR HPV-positive control (448 bp); lane 4, HPV-negative control; lane 5, second-step NPCR HPV-positive control (138 bp); lane 6, HPV-negative control; lanes 7 to 10, alternating first-step and second-step NPCR products from two HPV-positive specimens. **B**, amplification products with type-specific PCR primers. M, 100-bp DNA marker; lane 1, HPV 16-positive control (172 bp); lane 2, HPV-negative control; lane 3, HPV 16-positive specimen; lane 4, HPV 18-positive control (172 bp); lane 5, HPV-negative control; lane 6, HPV 18-positive specimen. **C**, representative restriction fragment length polymorphism of specimens with single HPV 16 infections. C, HPV 16-positive control; M, Φ $\times$ 174/*Hae*III DNA marker; lanes 2 to 4, HPV 16-positive specimens.

nin labeled oligonucleotide probes. Hybridization membranes were washed in posthybridization buffer (0.1% sodium dodecyl sulfate in 2 $\times$ standard

saline citrate) three times for 10 min at 55° C and in blocking solution (0.5% Tween 20, 1% blocking reagent in 1 × alkaline phosphatase buffer [AP] 7.5) for 45 min at room temperature. Signal was detected using antidigoxigenin-alkaline phosphatase conjugate diluted 1:5000 in blocking solution for 30 min. Finally, the membranes were washed in blocking solution 1 × AP 7.5 (0.1 M Tris, 0.1 M NaCl pH 7.5, and 10 mM MgCl₂) three times for 5 min and 1 × AP 9.6 (0.1 M Tris, 0.1 M NaCl pH 9.6, and 10 mM MgCl₂) and developed using nitroblue tetrazolium/bromo-chloro-indolyl phosphate in AP 9.6, up to 16 h. Water blank PCR products were used as negative controls (Fig. 2).

Nonisotopic *in situ* hybridization

Hybridization, labeling, and evaluation were performed as previously described (9). In an additional signal-enhancing step, labeling by biotinyl-tyramide was applied, according to the manufacturer's directions (TSA-Indirect, NENTM Life Science Products, AlterChem, Athens, Greece). Visualization was carried out using diaminobenzidine tetrahydrochloride (Sigma Hellois, Athens, Greece) (Fig. 3).

Statistical analysis

χ^2 tests with Yate's correction were performed using the Microstat software package (Ecosoft, Inc., Athens, Greece). Two-tailed Fisher's exact test was used when the samples in any cell were fewer than 5. *P* values lower than 0.05 were considered significant.

RESULTS

NPCR analysis detected the presence of HPV genome in 48 of 53 (90.6%) pathologic specimens examined. In contrast, none (0%) of the 16 normal samples were found to be infected (Table 2). Of the

FIGURE 2. Representative dot blotting of type-specific polymerase chain reaction products for HPV 16, 18, and 58. C₁, C₂, and C₃, positive controls for human papillomavirus (HPV) 16, 18, and 58, respectively. 1 to 3, HPV 16-positive cases; 4 to 6, HPV 18-positive cases; 7, HPV 58-positive case; N₁, N₂, and N₃, negative controls for HPV 16, 18, and 58, respectively.

FIGURE 3. Representative nonisotopic *in situ* hybridization analysis. A, punctate (type 2) signal in Caski cells (arrows). B, diffuse (type 1) in an oral hyperplasia infected by human papillomavirus 6. C, punctate (type 2) signal in an oral squamous cell carcinoma infected by human papillomavirus 16 (arrows) (original magnification, ×1000). Diaminobenzidine used as chromogen.

positive specimens, 34 (70.8%) were identified by the first step and 14 (29.2%) by the second step of the NPCR assay. This finding is suggestive of a viral load exceeding one copy per 10² cells in the former samples (48). HPV was detected in 25 of 29 hyperplasias (86.2%), 5 of 5 dysplasias (100%), and 18 of 19 SqCCs (94.7%). There was no correlation between HPV infection, histology (*P* = .46), and classic HPV changes. Positivity for HPV was also independent of cancer differentiation (*P* = .72).

HPV genotyping was performed by TS-PCR and DB. All infected specimens identified by NPCR were also positive by TS-PCR, which indicates that the number of viral DNA copies was always higher than one per 10^3 cells (48). Single HPV 6, 16, 18, and 33 infections were detected in 1 (2.1%), 24 (50.0%), 11 (22.9%), and 1 (2.1%) positive samples, respectively. Coinfections by types 6/16, 16/18, 16/33, 18/33, 16/18/33, 6/16/33, 6/16/18, and 6/11/16/18/31/33 were revealed in 3 (6.3%), 2 (4.2%), 1 (2.1%), 1 (2.1%), 1 (2.1%), 1 (2.1%), 1 (2.1%), and 1 (2.1%) positive lesions, respectively (Table 2). At least one high-risk type was detected in 47 (98.1%) infected specimens, with only 1 SqCC (1.9%) being infected solely by a low-risk viral type (HPV 6). HPV 16 was the prevailing type, being present in 34 (70.8%) infected cases either alone or in combination with other viral types. Single HPV 16 and HPV 18 infections were confirmed by RFLP analysis (Fig. 1). No association was observed between HPV typing and histology ($P = .14$). DB results (Fig. 2) were in agreement with those obtained by TS-PCR. Furthermore, DB analysis revealed the presence of HPV 58 in three (10.3%) hyperplastic specimens, also infected by types 6 and 16.

NISH confirmed the presence of HPV DNA in the 34 samples found to be positive by the first step of the NPCR assay. On the contrary, none of the 14 samples that were shown to be infected by the second step of NPCR were positive by NISH. This suggests that the number of viral copies in the latter specimens was lower than the detection limit of NISH (10 to 20 copies per cell) (55, 58). The physical status of the virus was evaluated before the tyramide amplification step. Granular (type 2) NISH signals, indicative of viral integration into the host genome, were obtained in lesions infected by high-risk viral types (56) (Fig. 3A, C). However, diffuse (type 1) signals were observed for low-risk types, suggesting an episomal HPV presence in infected cells (Fig. 3B).

In relation to further clinicopathologic features of the analyzed group of patients, statistical analysis revealed no correlation between HPV infection and gender ($P = .31$), age ($P = .49$), or smoking ($P = .14$). It is interesting that the percentage of HPV infection (90.6%) exceeded by far that of smoking (71.7%).

DISCUSSION

Reports on HPV involvement in oral carcinogenesis are conflicting, with infection percentages ranging from 0 to 87% (Table 1). The observed discrepancies are not fully justifiable by the epidemiologic differences of the examined patient groups or the selective investigation of certain types of oral proliferative conditions, but they may be

attributed to the varying sensitivity of the applied methodologies.

To exclude the methodologic variable, we used a highly sensitive NPCR assay as the basis of our experimental procedure. Amplification was performed by two sets of primers, targeted to the highly conserved 3' end of the L1 HPV gene, with the combined sensitivity of the first and second step of the assay theoretically reaching one viral copy per 10^6 to 10^7 cells (48, 50). We have analyzed a series of oral lesions representing the whole histologic spectrum of potentially neoplastic and neoplastic conditions, along with 16 oral samples derived from healthy individuals.

The infection percentage that we detected exceeds 90% in pathologic cases and is the highest ever reported. None of the examined normal samples were found to be positive, which lies in accordance with some studies (18, 23, 26, 29, 32). However, other groups have observed a surprisingly high infection percentage in normal mucosa (3, 11, 16, 41), possibly because they analyzed normal tissues that were adjacent to neoplastic lesions. In patients who had proliferative disorders, HPV positivity exceeded the percentage of smoking (71.7%), which is considered a major risk factor for oral carcinogenesis (3). Most studies agree that there is no statistically significant correlation between HPV prevalence and smoking history, although it seems that both tobacco and HPV infection may participate in the tumorigenic process in oral mucosa (13, 29, 34, 38, 43).

That the majority of positive lesions (70.8%) were revealed after the first PCR reaction is indicative of a high viral load and supports the biologic significance of our findings. In comparison to the literature, we have observed the highest infection percentages in every pathologic stage, although comparable infection percentages have been reported by others (10, 18, 21, 33, 37, 45). This finding suggests that the virus may be involved in early stages of oral carcinogenesis. As expected by the high prevalence of HPV in our patient group, no correlation was established between viral presence and other clinicopathologic characteristics (age, gender, and cancer differentiation). Such associations have been reported by some groups (13, 15, 38) but could not be confirmed by others (34, 43, 59). In addition, anatomic site seems to be an important factor that determines the susceptibility of squamous epithelium to HPV transformation, with the tonsillar region being more sensitive (13, 15, 22, 35). It is noteworthy that we observed such a high infection rate despite that no tonsillar carcinomas were included in our series of tissues.

TS-PCR revealed the presence of at least one high-risk type in 47 (98.1%) infected specimens. All infected samples identified by NPCR were also pos-

itive by TS-PCR, which suggests that the number of viral DNA copies was consistently higher than one per 10^3 cells (48). In accordance with the literature, the prevailing viral type was HPV 16, which was detected in 70.8% of positive samples, either in single infections (50.0%) or in coinfections (20.8%). Single HPV 16 and HPV 18 infections were confirmed by RFLP analysis. More than one high-risk type was co-detected in 11 (22.9%) specimens, whereas low-high-risk coinfections were observed in 6 (12.6%) cases. Both patterns of coinfection have been reported (Table 1). High-high-risk HPV infections may represent a synergistic oncogenic effect of the coinfecting types. Conversely, the low-high-risk infection pattern may be indicative of an incidental colonization of oral mucosa by the low-risk viral type and, hence, a solitary tumorigenic action of the high-risk type (9). Results of HPV positivity and typing in infected specimens were confirmed by DB. In addition, the presence of HPV 58 was detected by a specific probe in three (10.3%) hyperplastic lesions, always in combination with HPV 6 and 16. This is the first report of oral infection by this high-risk type.

In situ hybridization detected HPV DNA in all infected specimens that were identified by the first step of NPCR, suggesting a high viral load in these samples (56). NISH has generated signals that were characterized as punctate (type 2) in high-risk infections, whereas diffuse (type 1) signals were observed for low-risk types. Type 1 and 2 signals have been associated with the integrative and episomal pattern of HPV infection, respectively (60). Because biotinyl-tyramide enhancement sometimes renders this interpretation deceptive, the physical status of the virus was evaluated before signal amplification. In organs such as the larynx and the cervix, high-risk HPV infection is usually accompanied by viral integration into the host genome (7). It has been postulated that this integration is a critical event in the development of a malignant phenotype, because it allows persistent expression of E6 and E7 HPV genes. However, transcripts of these genes are detectable in episomal high-risk infection of oral keratinocytes *in vitro*, suggesting that viral integration is not essential for their expression (4). E6 protein binds to p53 and induces its degradation, whereas E7 binds to pRb and prevents its downstream activity. Functional deregulation of these vital cell growth regulators results in uncontrolled DNA replication and apoptotic impairment, which merely explains the increased oncogenic ability of high-risk types (7, 61).

In conclusion, the absence of HPV in normal samples, along with the high frequency of high-risk HPV types in the examined pathologic specimens, suggests an association of the virus with oral carcinogenesis. Furthermore, the high infection per-

centage in hyperplasias and dysplasias is indicative of an early involvement of HPV in oral neoplasia.

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Book Review

Rapley R, editor: *The Nucleic Acid Protocols Handbook*, 1050 pp, Totowa, NJ, Humana Press, 2000 (\$110).

This book contains 120 standard protocols for analyzing nucleic acids. It is divided into 10 parts, including nucleic acid extraction, probe design and synthesis, gene library construction and screening, DNA sequencing, polymerase chain reaction, and techniques for inducing and identifying mutations and for localizing genes. Almost all chapters contain references to Maniatis's 1989 spiral-bound volumes, indicating that the present book is in essence a practice-tested version of that classical laboratory manual. Most protocols refer to widely used techniques, the description of which could be found, in one form or another, in many other manuals or research

papers. The advantage to the potential user is that the techniques are presented in minute details and in a "cookbook-standardized manner." That most contributors are from England or Europe should not discourage anyone because the themes covered are universal. The personal notes inserted by each contributor provide assurance that the techniques would work in any laboratory, as long as the instructions are followed carefully. Having all of those protocols updated and tested in practice and bound in one volume will be attractive to many scientists who deal with molecular biology techniques.

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