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Original Paper

Detection of Human Papillomavirus DNA in Laryngeal Squamous Cell Carcinoma by Polymerase Chain Reaction

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Although human papillomaviruses (HPVs) have been found in many, but not all, tumours of the oral cavity, nose, pharynx and larynx, the true role of HPV in malignant tumours of the head and neck is still unclear. The presence of HPV DNA was investigated in 45 fresh squamous cell carcinoma (SCC) specimens and in 29 normal mucosa specimens collected from 45 primary laryngeal SCC patients. HPV DNA was detected using the polymerase chain reaction (PCR) with consensus primers that detect HPV types 6, 11, 16 and 18. 9 of the 45 patients (20%) were HPV positive; the presence of HPV was also detected in the corresponding normal laryngeal mucosa of four of the 29 specimens (14%). No statistically significant differences were found between the presence of HPV DNA in normal specimens and in neoplastic mucosa specimens. No correlation was found between HPV DNA positive tumours and size, T classification, lymph node involvement and histological grading. This study adds further evidence suggesting a possible role of HPV DNA infection in laryngeal carcinogenesis. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

THE AETIOLOGY of laryngeal cancer has yet to be clarified, despite its importance among head and neck malignancies. Laryngeal cancer accounts for almost 1.2% of all cancers in U.S.A. [1]. In Spain, France and Italy the incidence of laryngeal cancer is 10/100000/year, greater than in the U.K. where it is 4/100000/year [2]. Almost all laryngeal cancers (90–95%) are squamous cell carcinomas (SCC). Generally, the peak incidence is in the sixth and seventh decades of life [3], with a clearcut male preponderance, although this may change in the future due to increased smoking habits in females [4]. The predicted mortality is 32% with an overall survival rate of approximately 70% [5]. Although it is known that the main aetiological agents of laryngeal cancer are alcohol and tobacco abuse, other occupational and environmental factors (wood dust, rubber, gasoline fumes and paint, mustard gas, asbestos, nickel, sulphuric acid, hair dye) [6], are probably also involved. Limited evidence is available on the involvement and role of viral infections, and of human papillomaviruses (HPVs) in particlar, in the aetiology of laryngeal neoplasms. Nevertheless, some of the almost 60 types of HPVs have been detected in laryngeal papillomas [7] and in verrucous [8–11] and squamous cell carcinoma (SCC) [12–19] of the larynx. These data suggest that HPV infection, mainly from types 6, 11, 16, 18 and 30, might be closely associated with the development of some proliferative lesions. A synergistic effect of cofactors (viral, chemical, physical) has been shown in some malignancies, and is consistent with a multistage model for carcinogenesis. While strong evidence exists concerning the relationships of anogenital cancers and skin warts with HPV infections [20], the synergistic interaction of HPV DNA with chemical factors, especially cigarette smoking and alcohol consumption, is still a matter of discussion in the development of laryngeal cancer.

Although the spectrum of HPV-linked human cancers is widening, the role of such epitheliotrophic viruses in the aetiology of respiratory cancers is still unclear, as is the mode

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of transmission of respiratory HPV infection. Possible modes of transmission of HPVs to laryngeal mucosa are infection at birth, direct contact with infected individuals [21] and inhalation of vapourised material during laser removal of any virally induced neoplasm [22].

HPV-induced tumours are primarily benign and show only limited growth. Malignant conversion occurs occasionally and is usually associated with a loss of HPV regulation [23]. Moreover, virus types present in benign tumours are rarely found in carcinomas. HPV types 6 and 11, classified as low risk types, are commonly associated with solitary or multiple laryngeal papillomas. Nevertheless, HPV types 6 and 11 have also been detected in anogenital tumours and respiratory tract malignancies [24, 25]. HPV types 16 and 18 have been classified as high-risk types and identified in human carcinomas at various sites. The malignant progression of benign lesions induced by the low-risk HPV types may largely depend on synergistic exogenous carcinogens, as is evidenced by the development of carcinoma in laryngeal papillomas after radiation therapy [26]. Alternatively, the lesions associated with high-risk HPVs may progress to malignancies without such factors, as a consequence of virus-generated endogenous events [20]. The transforming activity of HPV seems to depend on the deregulated expression of E5, E6 and E7 HPV-oncoproteins and their ability to interact with some oncogene products. In particular, the E5 protein seems to interact with EGFR and may alter cell response to signals for growth and differentiation, while E6 and E7 proteins form complexes with cellular tumour suppressor proteins (pRB, p53) [27–29].

Immunohistochemical techniques have improved the detection rate of HPV in laryngeal SCC to between 10 and 20%, while modern polymerase chain reaction (PCR) analysis is even more sensitive, as it allows the detection of a higher percentage of HPV in SCC of the larynx.

The aim of this paper was to determine the prevalence of HPV types 6, 11, 16, 18 in normal and neoplastic laryngeal mucosa specimens and to correlate HPV presence with the epidemiological and clinicopathological characteristics of these patients.

PATIENTS AND METHODS

Patients

A series of 45 patients with primary laryngeal SCC, 42 males and 3 females, aged between 41 and 83 years (median 64 years), were admitted to our study. The clinicopathological features of the cases examined are shown in Table 1. 5 patients were non-smokers (4 males, 1 female) while 40 patients had a long-standing history (more than 20 years) of cigarette smoking. Of these, 35 had smoked more than 20 cigarettes per day (regular smokers) and 37 had consumed alcoholic beverages daily. 18 had been heavy drinkers (>1 litre/day of wine). Tumours were staged according to TNM classification (UICC, 1992) and graded as well (G1), moderately (G2) and poorly (G3) differentiated. A total of 74 specimens were collected at the time of surgical excision. With respect to the tumour site, 14 were supraglottic, 4 glottic and 27 transglottic. 19 patients underwent radical laryngectomy and 4 underwent radical pharyngolaryngectomy, while 21 had conservative surgery, i.e. 13 horizontal supraglottic laryngectomy, 4 cordectomy, 4 hemilaryngectomy; 12 had an associated neck dissection; 1 patient received radiotherapy alone and 8 postsurgical complementary radiotherapy.

Table 1. Clinicopathological features of 45 primary laryngeal SCC patients

		Smoke			Wine		
	N	≥20	<20	No	≥1 litre	<1 litre	No
Sex							
Males	42	35	2	5	17	18	7
Females	3	0	3	0	1	2	0
Age							
<60	16	15	1	0	3	13	0
>60	29	20	4	5	15	7	7
Site							
Glottic	4	1	1	2	1	1	2
Supraglottic	14	14	0	0	7	7	0
Transglottic	27	20	4	3	10	12	5
T							
T1	8	5	1	2	1	5	2
T2	20	16	2	2	13	5	2
T3	9	8	1	0	1	7	1
T4	8	6	1	1	3	3	2
N							
N0	24	17	2	5	8	10	6
N1	18	15	3	0	10	7	1
N2	3	3	0	0	0	3	0
Grading							
G1	3	1	0	2	0	1	2
G2	29	23	3	3	11	15	3
G3	13	11	2	0	7	4	2

Sample collection

Whenever possible, two biopsy specimens were obtained from each patient: one from the central portion of the tumour (all patients) and one from a non-tumour area of the larynx (29 out of 45 patients). Both tumoral and normal fresh tissue specimens were immediately frozen on dry ice shortly after surgical removal and stored at −70°C until HPV detection.

Sample preparation

Genomic DNA was extracted by the standard proteinase K/phenol method [30]. After ethanol precipitation, the DNA was resuspended in TE buffer (10 mM Tris–hydrochloride–1 mM EDTA) and quantified by measuring absorbance at 260 nm. Only DNA with a 260/280 ratio >1.8 were judged acceptable. DNA samples were then checked by agarose gel electrophoresis to verify that degradation had not occurred during the extractions. Aliquots of 10 μl (0.5–1 μg) of purified DNA were used for the PCR [31, 32], as reported below.

HPV DNA detection

HPV type 6, 11, 16, 18-specific "anticontamination" primers, flanking the plasmid cloning site to prevent amplification of contaminating cloned HPV types [33, 34], were used. Primer sequences, nucleotide localisation and amplified fragment length are shown in Table 2. PCR was carried out in 100 μl of reaction mixture containing 50 mM KCl, 1.5 mM $MgCl_2$, 25 pmol of each type-specific primer, 20 μM of deoxynucleoside triphosphate and 2.5 U of thermostable Taq DNA polymerase (Amplitaq, Perkin–Elmer, Cetus). Forty cycles of amplification were performed using a PCR processor (9600 Perkin–Elmer). Each cycle consisted of a

Table 2. Specification of oligonucleotide used as primers and probes for HPV detection with PCR

Group A primer	Nucleotide sequence	Region and nucleotide localisation	Amplimer length (base pair)
HPV 6-1	+5′ TAGTGGGCCTATGGCTCGTC 3′	E5 4671–4690	
			280
HPV 6-2	−5′ TCCATTAGCCTCCACGGGTG 3′	E5 4931–4950	
HPV 11-1	+5′ GGAATACATGCGCCATGTGG 3′	L1 6841–6860	
			360
HPV 11-2	−5′ CGAGCAGACGTCCGTCCTCG 3′	L1 7181–7200	
HPV 16-1	+5′ TGCTAGTGCTTATGCAGCAA 3′	L1 6028–6047	
			152
HPV 16-2	−5′ ATTTACTGCAACATTGGTAC 3′	L1 6160–6179	
HPV 18-1*	+5′ AAGGATGCTGCACCGGCTGA 3′	L1 6903–6922	
			216
HPV 18-2*	−5′ CACGCACACGCTTGGCAGGT 3′	L1 7100–7119	

Group B probe	Nucleotide sequence	Region and localisation
HPV 6	+5′ CATTAACGCAGGGGCGCCTGAAATTGTGCC 3′	E5 4761–4790
HPV 11	+5′ CGCCTCCACCAAATGGTACACTGGAGGATA 3′	L1 6977–7006
HPV 16	+5′ GCAAACCACCTATAGGGGAACACTGGGGCA 3′	L1 6117–6146
HPV 18	+5′ TGGTTCAGGCTGGATTGCGTCGCAAGCCCA 3′	L1 7021–7050

*HPV 18-specific primers are anticontamination primers for the Bam HI 6, 8 kb subfragment of pHPV18, used in our laboratory.

denaturation step at 94°C for 45 s, followed by primer annealing step at 55°C for 1 min and a chain elongation step at 72°C for 1.5 min. A 10 µl aliquot of the reaction mixture was analysed by 3% agarose gel electrophoresis (NuSieve, FMC) and ethidium bromide staining [30], and fragments of the expected length were identified based on their molecular weight. Known HPV-positive (HPV 16 DNA from Casky cell-line) and HPV-negative controls (human placental DNA) were utilised for all amplifications. Adequacy of DNA was checked by amplification of 50 pg (1 µl) of genomic HPV type 18 DNA from HeLa cell line added to each negative-sample DNA. Preliminary analysis revealed that no cross-amplification occurred with different types of primers. In all cases, final determination of specific amplification products was carried out by Southern-blot analysis. In order to verify if the HPV-negative DNA samples were suitable for PCR analysis, human beta-globin gene was amplified as control. Beta-globin-specific primers (Beta fw 5′ CAA CTT CAT CCA CGT TCA CC 3′, Beta rv 5′ GAA GAG CCA AGG ACA GGT AC 3′) were used in a separate reaction with aliquots of samples equal to those used in the HPV amplifications. Aliquots of amplified DNA were transferred from the gel to a Nylon membrane (Gene Screen Plus, Dupont) by diffusion blotting in denaturating conditions [35]. Membranes were hybridised with type-specific probes (Table 2) labelled with digoxigenin-dUTP using a DIG-oligonucleotide 3′-end labelling kit (Boehringer Mannheim) according to the manufacturer's protocols [34].

Statistical analysis

The Student *t*-test was used to analyse the presence of HPV DNA in both normal and neoplastic tissue, in relation to size, T classification, lymph-node involvement, and histopathological grading of the tumours.

RESULTS

Human papillomavirus DNA was detected in 9 (20%) of the 45 patients (Figures 1–3). All 9 were positive for HPV type 16 while 2 (cases 2 and 5) contained both HPV type 6 and 16. HPV type 16 was also detected in three and HPV type 6 in one of the normal laryngeal mucosa specimens (Table 3). HPV type 18 was not detected. Four (44%) of the nine HPV-positive tumours were supraglottic and five (56%) transglottic. 45% of the HPV-positive tumours were T3, 33% were T4 and the remaining 22% were T1–T2. Fifty-five per cent of the HPV-positive cancer patients had no clinical cervical lymph nodes (N0), 33% were N1 and 22% were N2. Thirty-three per cent of HPV-positive tumours were G3 (poorly differentiated), 55% were G2 (moderately differentiated) and the remaining 22% were G1 (well differentiated). All HPV-positive cancer patients were daily cigarette smokers and 4 consumed more than a litre of alcohol per day. The successful amplification of the 268 bp fragment of human beta-globin

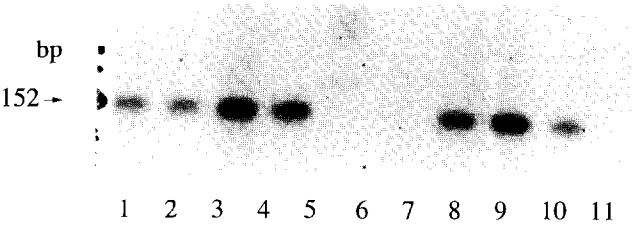

Figure 2. Southern blot analysis of PCR products with labelled HPV type 16-specific oligonucleotide probe. Nucleotide sequence lengths indicated are sized by DNA marker VI and DNA marker IX (Boehringer Mannheim). Lane 1: HPV 16-positive control. Lanes 2, 3, 4, 8, 9 and 10: HPV 16-positive specimens from SCC patients. Lanes 5, 6 and 7: HPV 16-negative specimens from SCC patients. Lane 11: negative control (human placental DNA).

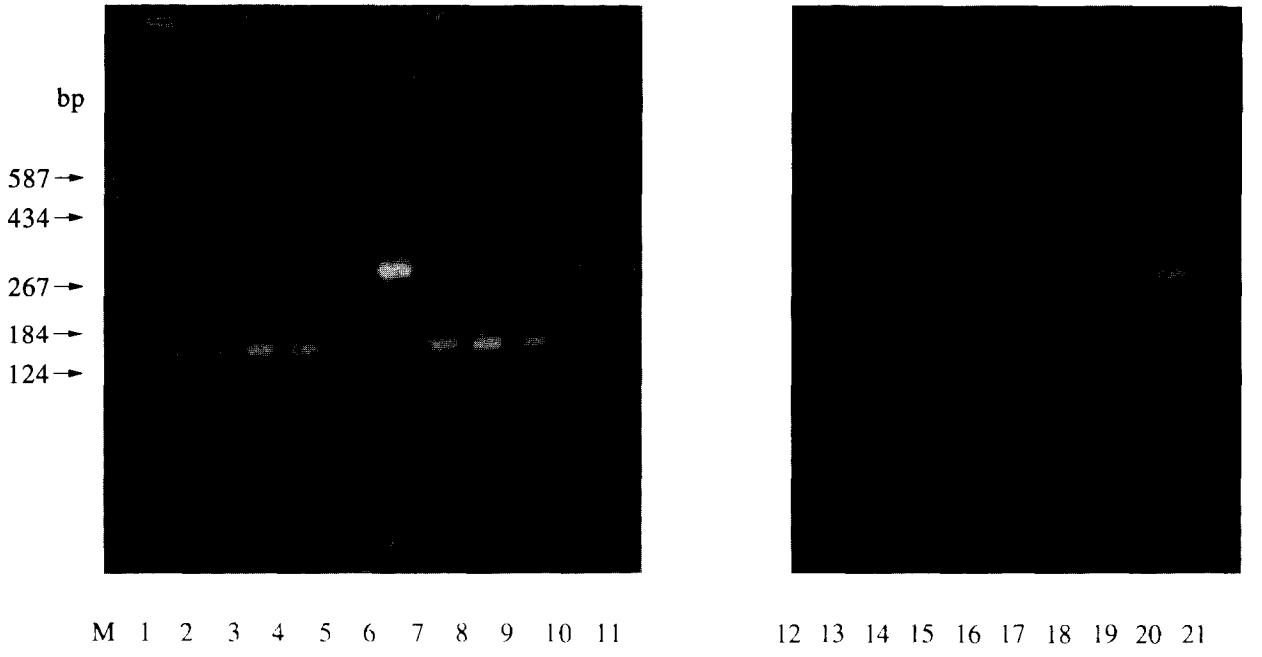

Figure 1. Detection of HPV DNA in human biopsies by PCR. Amplification products after gel electrophoresis and ethidium bromide staining. Lane M: DNA size marker (marker VI Boehringer Mannheim). Lane 1: HPV 16-positive control (DNA from human CasKi cell line). Lanes 2, 3, 7, 8, 11, 14 and 16: 7 cases of HPV16-positive SCC patients. Lanes 4 and 5 (case 2) and 9 and 10 (case 5): 2 cases of HPV 16- and HPV 6-positive SCC patients. Lane 6: HPV6-positive normal mucosa from patient 4 (SCC mucosa HPV16-positive – lane 7). Lanes 12, 13, 15, 17, 18 and 19: 6 cases of HPV-negative SCC patients. Lane 20: HPV 6-positive control (human DNA from HPV 6-positive cervical specimen). Lane 21: negative control (human placental DNA).

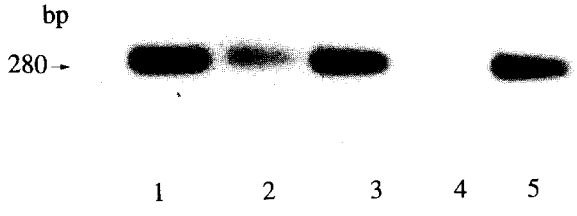

Figure 3. Southern blot analysis of PCR products with labelled HPV 6-specific oligonucleotide probe. Nucleotide sequence lengths indicated are sized by DNA marker VI and DNA marker IX (Boehringer Mannheim). Lane 1: HPV 6-positive control. Lanes 2, 3 and 5: HPV 6-positive specimens from SCC patients. Lane 4: negative control (human placental DNA).

Table 3. Detection of HPV types 16 and 6 by PCR analysis in laryngeal SCC and corresponding normal mucosa specimens from 9/45 patients

Case	Age	Sex	SCC	NM
1	68	M	16	—
2	60	M	16,6	16
3	74	M	16	–
4	60	M	16	6
5	61	M	16,6	16
6	52	M	16	—
7	68	M	16	—
8	71	M	16	16
9	57	M	16	—

NM, normal mucosa; SCC, squamous cell carcinoma.

gene from all HPV-negative DNA samples showed that DNAs were sufficiently intact and no PCR inhibitors were present (Figure 4). This control analysis shows that negative biopsies were truly viral negative samples. No differences were found between HPV-positive and HPV-negative cancer patients with regard to age, occupational risk, or alcohol consumption, but all HPV-positive patients had a long-standing history of cigarette smoking. No statistically significant differences were found between the presence of HPV DNA in normal and neoplastic mucosa specimens. HPV DNA-positive tumours did not correlate with size, T classification, lymph node involvement and histological grading.

DISCUSSION

Until recently, several techniques have been used to detect the presence of HPV DNA. Southern-blot analysis, previously

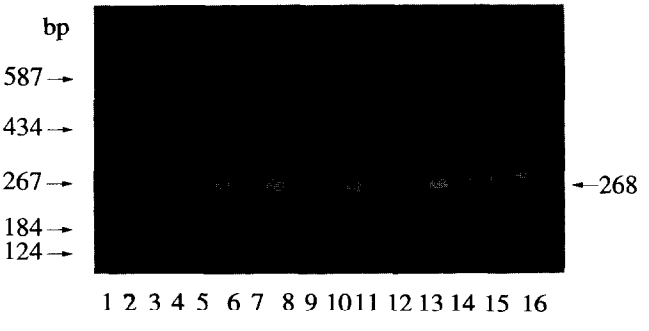

Figure 4. Amplification of the beta-globin fragment (268 bp) from 14 human biopsy samples negative for HPV DNA after electrophoresis on 1% agarose gel and ethidium bromide staining. Lane 1: DNA size marker (Marker V, Boehringer Mannheim). Lane 2: negative control (human placental DNA). Lanes 3–16: beta-globin-positive human biopsy samples.

considered to be the most sensitive assay, is highly complex and labour intensive. A simpler technique, the Dot-blot assay, has a low sensitivity, as does *in situ* hybridisation analysis, which can be performed on paraffin-embedded sections. All these techniques have the disadvantage that they require a sizable quantity of nucleic acids and take several days to 2 weeks to perform.

PCR is a recently developed technique for *in vitro* amplification of specific targets (DNA or RNA sequences), which are amplified to readily detectable levels by electrophoresis and subsequent dot-blot hybridisation. This technique does not require a large amount of sample material, and can detect HPV DNA sequences in formalin-fixed or paraffin-embedded tissues, in addition to fresh tissue. Results can be available within 24 h. PCR is more sensitive than Southern-blot analysis [36] but its high sensitivity may lead to product carryover or DNA contamination, which can create problems in diagnostic applications. Anticontamination primers were used to avoid this artificial HPV DNA detection and to eliminate false-positive results. To our knowledge, no papers concerning the use of PCR in the detection of HPV DNA in fresh laryngeal SCC and normal mucosa have been published so far.

Recent data have linked HPV types 16 and 18 to various squamous cell carcinomas in the oral cavity [13, 15, 37], tongue [38, 39], pharynx [18], tonsil and maxillary sinus [14, 40]. The presence of HPV types 6, 16, 18 and 30 has been observed in up to 10% of laryngeal SSC by various authors using Southern blotting or *in situ* hybridisation [12, 14, 39, 43], while type 16 has been detected in verrucous carcinoma [8, 9].

In contrast, figures ranging from 8 to 54% have also been reported in the literature [16–19, 44]. Our results (20%) add further information to the previous HPV DNA studies of laryngeal SCC. We were able to detect the presence of HPV 16 DNA in 9 of the 45 cases of primary laryngeal SCC (20%), a percentage similar to the figures reported by other authors employing the Southern blotting technique, and by Ogura and associates [18] and Brandwein and associates [19] employing PCR analysis.

We failed to detect HPV type 18 in laryngeal SCC in agreement with the more frequent association of HPV type 18 infection in cancer of other human tracts, such as anogenital cancers and, in particular, adenocarcinomas of the cervix [45]. Moreover, our data confirm the possibility of detecting HPV type 6 in laryngeal carcinomas, in addition to the most common types 16, 18 and 30, and is in line with results reported by Hoshikawa and associates [17], Deckmezian and associates [13] and Syrjanen and associates [14].

Concerning the association between tumour site and HPV infection, our data do not confirm previous observations that laryngeal glottic SCCs showed higher HPV 16 DNA positivity than do supraglottic ones [17].

Some authors claim that HPV DNA is more frequently detected in well-differentiated [15, 40, 46] or in poorly differentiated SCC [44] of the upper aerodigestive tract. In our study, HPV DNA was detected more often in moderately and poorly differentiated SCC than in well-differentiated tumours. In our study, 4/29 (14%) normal laryngeal mucosa specimens obtained from sites adjacent to the tumour were positive for HPV, 3 for HPV type 16, and 1 for HPV type 6. Previous studies have paid little attention to the simultaneous detection of HPV DNA in both tumor specimens and corresponding normal tissue specimens. Brandsma and Abramson [43] found a prevalence of 4% of HPV DNA infection in normal laryngeal mucosa specimens, but these specimens were collected from non-cancer control patients. To date, data available in the literature are insufficient to exclude or to confirm a role of HPVs in laryngeal carcinogenesis. Nevertheless, based upon the data presented here, the HPVs could represent one of the factors in the development of laryngeal cancer. Furthermore, their detection in normal laryngeal mucosa of laryngeal cancer patients could suggest the hypothesis that long term presence of HPVs in the mucosa of normal patients could represent a risk factor for laryngeal cancer development. This cannot be dismissed considering the established causative role of HPVs in laryngeal papillomatosis [14], which may occasionally degenerate in SCC [47, 48], as well as in the multistep process of malignant progression [45].

The role of HPV in laryngeal carcinogenesis has not been demonstrated. The present findings support the hypothesis that laryngeal cancer is induced by exposure to HPV and other environmental factors, such as chemical tobacco-related carcinogens. Studies are in progress using Southern blot analysis after Hind III endonuclease digestion to analyse whether HPV DNAs found in laryngeal SCC are integrated or in episomal form. Further investigations are necessary to demonstrate the possible association between HPV-oncoprotein expression and some oncogene product alterations, in order to clarify their synergistic mechanism in the multifactorial development of SCC of the larynx.

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