The Journal of Laryngology and Otology
August 1996, Vol. **110**, pp. 742–747

Human papillomavirus and p53 oncoprotein in verrucous carcinoma of the larynx

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Abstract

The incidence of p53 antigen and human papillomavirus (HPV) expression in archival formalin-fixed, paraffin-embedded tissue sections from verrucous carcinoma of the larynx was determined using immunohistochemistry.

The p53 oncoprotein was detected in four of 10 tissue samples (40 per cent). The same number of tumours had HPV antigen, and three cases had both p53 oncoprotein and HPV antigen. All positive cases were from heavy smokers and drinkers.

After surgical treatment, no tumour recurrence was present in our series. Four patients developed a second head and neck neoplasm and death occurred in three. Three of the patients with second tumour had p53 positive immunoreactivity and two had p53 and HPV expression.

Verrucous carcinoma of the larynx presented with overexpression of p53 antigen in a similar percentage to other head and neck cancers. The p53 immunohistochemical determination is well correlated with HPV detection and could have prognostic value in these tumours, but no statistical evidence was present.

Key words: Laryngeal neoplasms; Immunohistochemistry; Papillomaviruses; DNA probes, HPV

Introduction

Verrucous carcinoma is a highly differentiated squamous cell carcinoma with a special clinical course of slow growth, local invasion, no metastatic capability and with a benign-appearing histology (Ferlito and Recher, 1980).

There is increasing evidence of human papillomaviruses (HPV) as aetiological agents of verrucous carcinoma following detection of HPV in the lesions (Mounts *et al.*, 1982; Weber *et al.*, 1988). Recently Kasperbauer *et al.* (1993) demonstrated that HPV DNA was present in the majority of a series of 20 verrucous laryngeal carcinomas. As noted for squamous carcinoma, the HPV may act as a cofactor in tumorigenesis in concert with other agents including tobacco, alcohol, irradiation, other viruses, or altered immune surveillance (Noble-Topham *et al.*, 1993).

Oncogenes and tumour suppressor genes play an important role in the initiation and progression of most neoplasms, of particular interest has been the potential role played by the p53 tumour suppression gene (Pavelic *et al.*, 1983). The p53 gene encodes a nuclear phosphoprotein thought to regulate proliferation of normal cells. Alterations of the p53 gene have been correlated with the genesis of many types of human cancers. In essence, the wild-type p53 gene participates in suppressing cell transformation, while the mutant alleles favour cell growth (Field *et al.*, 1993).

The normal p53 protein has a very short half-life (six to 20 minutes) but mutant forms have half-lives of up to six hours, thus detection of the p53 protein is synonymous with a mutation (Lane and Benchimol, 1990).

The p53 protein expression is defined by immunohistochemistry. This expression has been associated with a history of heavy smoking and drinking, and could be considered a poor prognostic factor in some tumours such as breast cancer (Davidoff *et al.*, 1991) and colorectal carcinomas (Sun *et al.*, 1992). Overexpression of p53 protein was also found in laryngeal papillomas (Clark *et al.*, 1993) and squamous cell carcinomas of the head and neck (Dolcetti *et al.*, 1992; Pavelic *et al.*, 1993), but there is no agreement about clinicopathological correlation and the prognostic value of this test in the head and neck. No previous studies have been reported in the English literature on p53 overexpression in laryngeal verrucous carcinoma.

The aims of the present study were to evaluate the incidence of p53 expression and HPV antigen determination by immunohistochemistry, in a group

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Presented at the National Meeting of the Spanish Society of Otolaryngology, Madrid, November 12, 1994.
Accepted for publication: 26 April 1996.

TABLE I
MAIN FEATURES OF VERRUCOUS CARCINOMAS IN THIS SERIES

Case no.	Age	Location	Size	Prior papilloma	Heavy smoking	Heavy drinking	Prior ENT cancer
1	59	Glottis	Small (T_1)	No	Yes	Yes	No
2	66	Glottis	Large (T_3)	Yes	Yes	No	No
3	63	Supraglottis	Large (T_3)	No	Yes	Yes	Yes (oral cavity)
4	69	Glottis	Large (T_3)	Yes	Yes	Yes	Yes (tongue base)
5	38	Supraglottis	Large (T_4)	No	Yes	Yes	No
6	67	Glottis	Large (T_3)	No	Yes	No	No
7	71	Glottis	Small (T_1)	No	No	No	No
8	70	Glottis	Small (T_2)	No	No	No	No
9	61	Glottis	Large (T_3)	No	Yes	Yes	No
10	62	Glottis	Small (T_1)	Yes	Yes	Yes	No

of verrucous carcinomas of the larynx, and to analyse their correlation with clinical data of the patients.

Materials and methods

Patients

Paraffin blocks from all the patients with primary verrucous carcinoma of the larynx treated with surgery at our hospital over a 20 year period (1973–1992) were collected from the tissue archives at the General Hospital of Galicia, Santiago Compostela, Spain. No patient had received previous treatment at our institution or elsewhere and no exclusions were made. Surgery was the only treatment employed. Patients were followed up after surgery until death, or for a minimum of 30 months.

All patients were male. Their ages ranged from 38 to 71 years (median = 62.6 years). Eight patients had a history of heavy smoking and six of heavy drinking. Three cases had a history of previous laryngeal papilloma. The papilloma histology was reviewed and confirmed. The time interval between the diagnosis of these papillomata and of verrucous carcinoma ranged from 27 to 66 months (median = 49 months). Two patients had previous head and neck squamous cell carcinomas. A laryngeal verrucous carcinoma was diagnosis 11 months after a tongue base carcinoma and another one 28 months after an oral cavity carcinoma. Both cases were treated with surgery and post-operative radiation therapy (dose = 5000 cGy).

Most of the verrucous carcinomas were located in the glottic region. The main characteristics of verrucous carcinomas are summarized in Table I.

Tissue specimens

Tissues fixed in 10 per cent neutral buffered formalin and processed for routine surgical pathology evaluation were cut as 5-μm sections and mounted on 3-aminopropyl-triethoxisilane-coated slides.

Pathology examination

Routine pathology evaluation was performed in haematoxylin and eosin stained sections, diagnosis was confirmed and the main histological characteristics, including presence of koilocytic cells, were analysed.

Immunohistochemistry

After blocking endogenous peroxidase activity with three per cent H_2O_2 for 10 minutes, serial sections of the tumours were incubated for one hour with monoclonal antibody against p53 (Biogenex Laboratories, San Ramon, California) at a dilution of 1:20 in PBS, or polyclonal antibody to HPV (Dako Corp., Carpenteria, California) diluted 1:2,000 in PBS. The secondary antibody in the first case was biotinylated anti-mouse Ig (Vector Laboratories Inc., Burlingame, California) and in the second one biotinylated anti-rabbit Ig (Vector Laboratories Inc., Burlingame, California). Both biotinylated antibodies were diluted at 1:100 and incubated during 30 minutes. The detection system employed was avidin biotin peroxidase complex (Kit Elite Vectastain, Vector Laboratories Inc., Burlingame, California) incubated for 30 minutes, followed by a 0.06% (W/v) solution of 3-3′ diaminobenzidine-tetrahydrochloride (Sigma) with 0.003 (v/v) hydrogen peroxide for 10 minutes. Negative controls were performed: (1) by substitution of primary antibody for non-immune serum of the same animal species of primary antibody, or (2) by alternative stratum suppression of any of reagents of the detection system. In both cases results were negative.

Evaluation of slides

Slides were examined for the presence of nuclear staining within the tumour itself. If any nuclear staining was seen, the tumour was considered positive for p53 or HPV.

Data analysis

Results for p53 and HPV were evaluated in relation to the smoking and drinking habits of the patient, prior squamous cell papillomas, location and size (T_1 and T_2 = small; T_3 and T_4 = large) of the tumour, presence of koilocytic cells and occurrence of a second primary head and neck cancer.

Observed differences were analysed for statistical significance by the Fisher and Chi squared tests, with

https://doi.org/10.1017/S0022215100134851 Published online by Cambridge University Press

TABLE II
IMMUNOHISTOCHEMICAL RESULTS, KOILOCYTOSIS AND SECOND HEAD AND NECK TUMOURS AFTER VERRUCOUS CARCINOMA OF THE LARYNX

Case no.	HPV	P53	Koilocytosis	Second larynx tumour	Other second ENT tumour
1	+	−	Yes	No	No
2	−	−	Yes	Yes (Squamous cell)	No
3	+	+	No	Yes (Squamous cell)	No
4	+	+	Yes	No	Yes (Squamous cell)
5	+	+	Yes	No	No
6	−	−	Yes	No	No
7	−	−	No	No	No
8	−	−	No	No	No
9	−	−	No	No	No
10	−	+	Yes	Yes (Spindle cell)	No

Yates correction. Differences lower than 0.05 were considered significant.

Results

Clinical data

No recurrence of a verrucous laryngeal carcinomas occurred. Four patients (40 per cent) developed a second malignancy in the head and neck, during the follow-up period. In three of these cases the second neoplasm was sited in the glottic region (two squamous cell carcinomas and one spindle cell carcinoma). The original site of the verrucous carcinoma was the glottis in two patients, but one of these patients had a spindle cell carcinoma in the contralateral vocal cord (patient number 10). The time interval between verrucous carcinoma and the second laryngeal tumour ranged from 17 to 26 months (median = 21.7 months).

The other case of secondary neoplasm was a squamous cell carcinoma of the hard palate, eight months after the diagnosis of laryngeal verrucous carcinoma.

No death related to a verrucous carcinoma occurred in our study, but three of the four patients with a second malignancy died.

All cases with squamous cell papilloma of the larynx before the verrucous carcinoma presented with a second primary head and neck tumour. This incidence showed a statistically significant difference compared with the group without previous papilloma ($p = 0.0333$).

Immunohistochemical data (Table II)

HPV staining (Figure 1) was present in four samples of verrucous laryngeal carcinoma (40 per cent), and the same number of tumours had p53

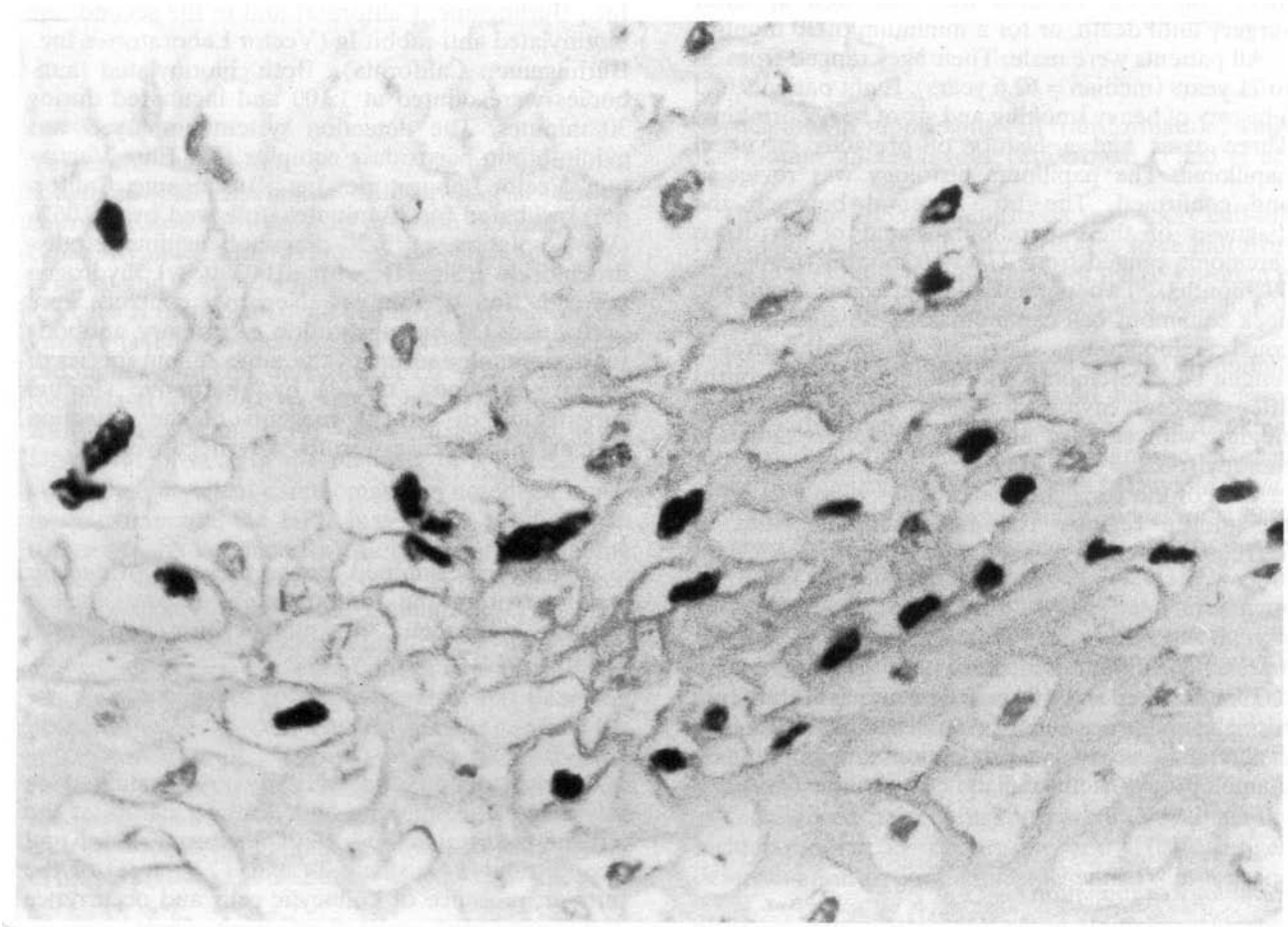

FIG. 1
Photomicrograph showing positive immunostaining for HPV (Immunohistochemistry; original magnification ×400).

https://doi.org/10.1017/S0022215100134851 Published online by Cambridge University Press

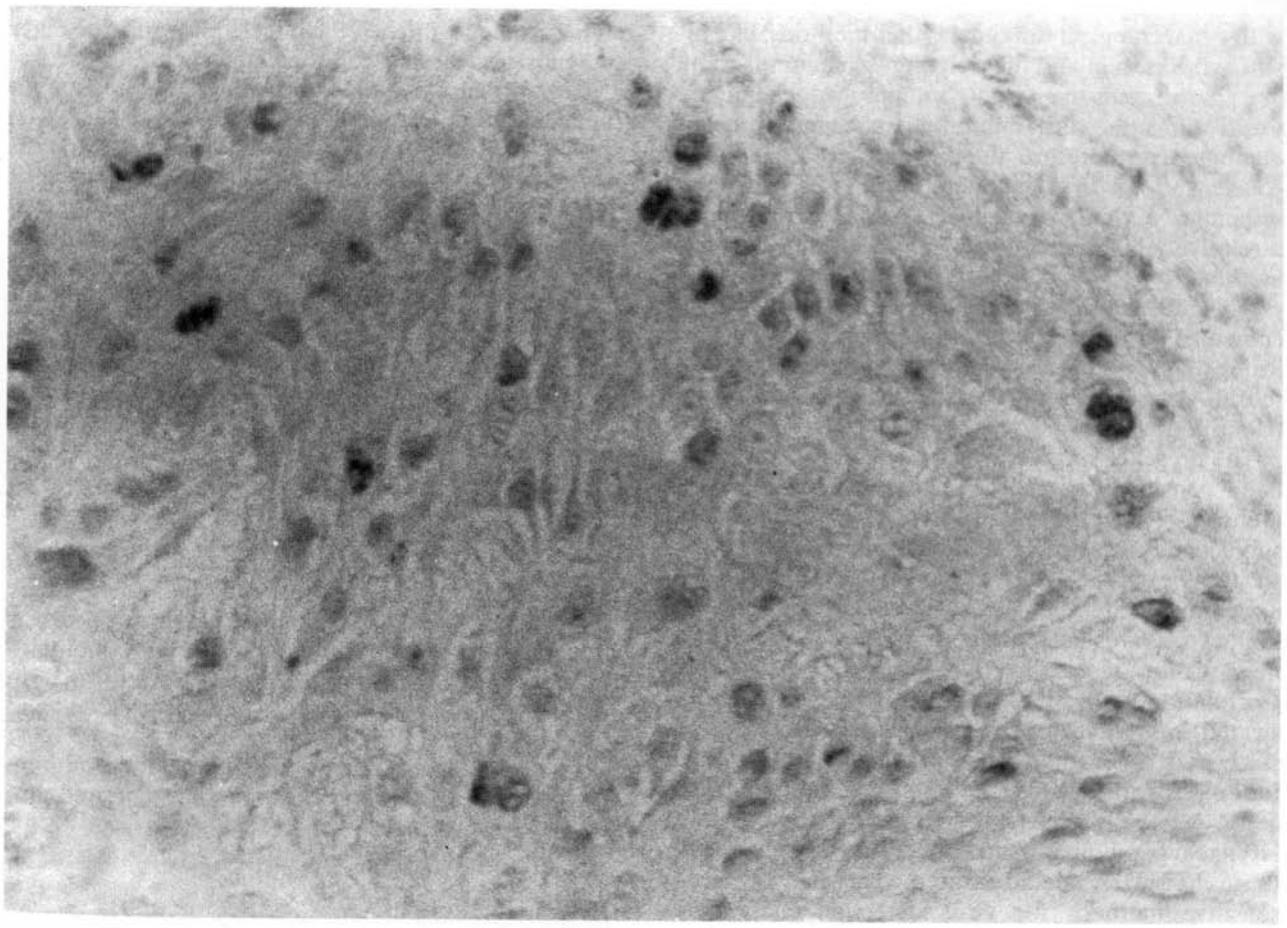

FIG. 2
Photomicrograph showing expression of p53 oncoprotein (Immunohistochemistry: original magnification ×400).

protein-positive (Figure 2) immunoreactivity. Three tumours had both HPV and p53 staining.

All patients with positive tests had a history of heavy smoking and drinking, and three of the four had a large tumour (T_3 or T_4).

Only one patient with previous laryngeal papilloma showed positivity for HPV. All patients with a history of previous head and neck carcinoma had positive HPV immunostaining. Two patients with previous cancer and two with a history of laryngeal papilloma had positive p53 staining.

Koilocytosis was demonstrated in six verrucous carcinoma samples. Koilocytic cells were found in three of four positive cases with p53 immunoreactivity, and the same percentage in tumours with HPV staining.

Three of the four cases with a second head and neck malignancy had p53 staining and two had HPV immunoreactivity.

No statistical significance was found between the differences observed in groups of patients with and without positive p53 or HPV staining.

Discussion

Verrucous carcinoma of the larynx is a variety of epidermoid carcinoma highly associated with smoking (Ferlito and Recher, 1980; Lundgren *et al.*, 1986) and HPV infection (Abramson *et al.*, 1985; Hagen *et al.*, 1993; Noble-Topham *et al.*, 1993). The tumour has a different behaviour when compared with other types of cancer, with local invasion, but no metastatic capability, and a tendency to anaplastic degeneration after radiotherapy (Hagen *et al.*, 1993).

In our series we found previous squamous papilloma of the larynx in 30 per cent of the cases; this entity having a similar viral aetiology (Mounts *et al*, 1982). Differential diagnosis between verrucous carcinoma and papilloma can be difficult, and inadequate biopsy of a verrucous laryngeal carcinoma can be misdiagnosed as a squamous papilloma, even on repeat biopsy (Ferlito and Recher, 1980). In our series, the time interval between the diagnosis of these papillomas and verrucous carcinoma was very long, a disease-free interval was present in all cases and the diagnosis of papillomas was confirmed.

HPV is well recognized as an aetiological factor for verrucous carcinoma. There are different HPV antigen detection methods for formalin-fixed, paraffin-embedded tissue sections. The immunohistochemical procedure, with the avidin-biotin-peroxidase complex, employed in the present study, has the advantages of being widely available and fast and cheap; nevertheless its sensitivity is relatively low and needs a high concentration of viral antigen in the sample (Levi *et al.*, 1989). This is a serious problem in lesions of the larynx where the concentration of HPV antigen is very low compared

with gynaecological tumours. Other methods, like in-situ hybridisation and the polymerase chain reaction, are more sensitive than immunohistochemistry. Positive results with immunohistochemical probes demonstrate, therefore, the presence of HPV in high concentrations in the tissue and it is a useful screening method. We found HPV DNA present in a relatively high percentage (40 per cent) using this method.

No statistical significance was found in the clinical course of patients related to the immunohistochemical results, due to the small number of cases, but we found very interesting results which need to be confirmed with larger series.

The presence of koilocytic changes have classically been considered a characteristic of virus infection (Jenson *et al.*, 1991). However, this diagnosis is subjective and also HPV antigen has been demonstrated in tissues with a morphologically normal appearance (Steinberg *et al.*, 1988). We found that koilocytosis was very frequent in verrucous carcinomas of the larynx in our series (60 per cent). Correlation between koilocytosis and HPV demonstrated immunohistochemically was present in this study as in other reports (Crum *et al.*, 1983). Three of four cases with positive HPV staining had koilocytic cells. All the verrucous carcinomas with previous laryngeal papillomas had koilocytosis but only one of these had HPV demonstrated. The use of more sensitive methods for virus detection, as *in situ* hybridization or the polymerase chain reaction, could increase the positive results, but the aim of this study was to evaluate the role of immunohistochemistry.

Patients with previous head and neck malignancies had positive staining for HPV. These results suggest that the viral infection is related to cancer pathogenesis (Noble-Topham *et al.*, 1993).

The immunohistochemical detection of p53 protein is an easy, fast and inexpensive method. The p53 overexpression was found in patients with a history of heavy drinking and smoking in a great number of lesions (Field *et al.*, 1992; Field *et al.*, 1993) and all positive cases in our series had a similar history.

We found a high correlation between positive results for p53 and HPV by immunohistochemistry, suggesting the presence of a relationship between p53 mutation and viral infection in carcinogenesis, in addition to genetic changes, tobacco and alcohol.

The prognosis of verrucous carcinoma is very good and very little mortality is reported in most of the published series (Hagen *et al.*, 1993). Some authors report that the presence of a second neoplasm is not increased in these patients compared with other otolaryngological tumours (Ferlito and Recher, 1980; Dolcetti *et al.*, 1992), but in our series a second cancer occurred in 40 per cent of patients and was the most important prognostic factor, as three patients died due to this second malignancy.

All cases with prior papilloma and/or head and neck cancer developed a second neoplasm. These observations suggest that patients with these tumours need intensive clinical follow-up for early diagnosis of these second malignancies.

The prognostic value of p53 overexpression in head and neck cancer is in doubt (Leedy *et al.*, 1994). In our series three of four p53 positive patients presented a second tumour. In spite of the lack of statistical evidence, the p53 determination, in addition to clinical antecedents and HPV determination, could be a useful method for the early detection of the high risk group in patients with verrucous carcinoma of the larynx.

Conclusions

Verrucous carcinoma of the larynx presented overexpression of p53 antigen in a similar percentage to other head and neck cancers. HPV immunohistochemical staining and koilocytosis were also very frequent in these tumours. The patients in the series had a high incidence of second head and neck malignancies. The p53 immunohistochemical determination is well correlated with the HPV immunohistochemical detection. Both tests were positive in patients with a history of heavy drinking and smoking.

The prognostic value of p53 staining for evolution to second head and neck tumours is suggested, but no statistical evidence was present.

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