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Human papilloma viruses: a study of their prevalence in the normal larynx

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Abstract

The association of human papilloma viruses (HPV) with laryngopharyngeal squamous cell carcinoma is under investigation. The suitability of control tissue in the reported series, invariably obtained from histologically normal tissue adjacent to a squamous cell carcinoma or from patients with benign laryngopharyngeal disease, is questionable. The present study determined the prevalence of HPV in a series of normal larynges.

Twelve autopsy larynges were collected. DNA was obtained by SDS proteinase K digestion. Evidence of HPV infection was documented by the polymerase chain reaction using oligonulceotide primers complementary to sequences in the E6 region of HPV types 11, 16 and 18.

Four female and eight male larynges, mean age 65 years (SD=16 years) were collected 72 hours postmortem (median value). HPV type 11 was isolated from three specimens. A 25 per cent prevalence rate for HPV 11 was found. No other HPV types were isolated.

Key words: DNA; Larynx; Polymerase chain reaction; Viruses

Introduction

Human papilloma viruses (HPV) types 6 and 11 have an established aetiological role in laryngeal papillomatosis (Terry *et al.*, 1987). The oncogenic potential of HPV in the human larynx is being investigated (Brandasma and Abramson, 1989; Morgan *et al.*, 1991).

The prevalence of HPV in laryngeal verrucous carcinoma, carcinoma *in situ* and invasive squamous cell carcinoma varies from five to 88 per cent; 6, 11, 16 and 33 being the subtypes isolated (Kashima, *et al.*, 1986; Dekmezian *et al.*, 1987; Brandasma and Abramson, 1989; Bryan *et al.*, 1990; Morgan *et al.*, 1991). Recent work suggests that the isolation of HPV is no greater in the carcinomatous larynx than in the control larynx, thereby questioning its aetological significance (Brandasma and Abramson, 1989; Bryan *et al.*, 1990).

Reported control tissue consists of biopsy specimens from adjacent nondiseased sites, or biopsy specimens taken from patients undergoing direct laryngoscopy in whom there is no evidence of respiratory papillomatosis or malignancy. The controls therefore invariably consist of tissue obtained from a group of patients with laryngeal pathology, albeit sometimes benign, and are unlikely to be representative of the general population.

A number of techniques have been used for the identification of HPV in the larynx which vary in their sensitivity and specificity. The polymerase chain reaction (PCR), the most sensitive available technique (Bryan *et al.*, 1990; Morgan *et al.*, 1991) was used to determine the prevalence of HPV in this series of normal autopsy larynges.

Methods

Larynges appearing normal were collected at autopsy with local ethical committee approval. The supraglottis, glottis and subglottis of each larynx was sampled. HPV sequences were detected by PCR using oligonucleotide primers complementary to the sequences of the E6 region of HPV types 11, 16 and 18 as described previously (Griffin *et al.*, 1990) In brief 4 × 10 μm paraffin sections were cut using a microtome, slide mounted and left on a hot plate for five minutes. The tissue was dewaxed in xylene, rehydrated through graded alcohols to water, and then scraped off into a 1.5 ml microcentrifuge tube to be incubated in 100–500 μl of digestion buffer containing 10 mM tris-hydroxymethylaminomethane hydrochloride (TRIS-HCL(pH 8.8, 100 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 0.5 per cent sodium dodecyl sulphate (SDS) and 2 mg/ml proteinase K at 37°C for five days. The aqueous digest was then extracted once with phenol:chloroform (1:1), once with chloroform:isoamyl alcohol (24:1) and the DNA was precipitated with two volumes of ethanol and 1/10 volume of 3 M sodium acetate at –20°C overnight. The DNA was collected after centrifugation at 15000 g for 10 minutes, dried and redissolved in 50 μl of ultrapure water: 10 μl aliquots were

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amplified to detect the presence of beta-globin before proceeding to HPV detection.

HPV detection was carried out using a reaction mixture containing 1 nM of each primer pair, 200 μM of each dNTP, 10 × reaction buffer (10 mM TRIS-HCL, pH 8.3; 50 mM KCl; 7.5 mM $MgCl_2$; 0.01 per cent gelatin; 0.1 per cent TRITON × 100; 1.0 unit of Taq polymerase (Super Taq: HT Biotechnology Ltd) in a total reaction volume of 50 μl. A positive control containing a HPV recombinant plasmid and a negative control in which ultrapure water was substituted for the aliquoted DNA were also amplified for each series of DNA specimens tested.

Temperature cycling was performed using a programmable cycler (M.J. Research, Waterdown, MA) programmed to perform one cycle at 94°C for five minutes, 55°C for 90 s, 72°C for two minutes and 39 cycles at 94°C for 30 s, 55°C for 90 s and 72°C for two minutes. The amplified products were visualized under ultraviolet light after electrophoresis for one hour on a two per cent agarose gel.

Stringent laboratory conditions were routinely used to reduce the risk of cross-contamination and false positivity. Furthermore the consistency of the findings were confirmed by repeating the PCR test protocols for all specimens on a minimum of two occasions, one week apart.

Results

Four female and eight male larynges were studied. The patients had a mean age of 65 years (SD=16 years). The larynges were harvested at a median time of 72 hours postmortem (range 24–144 hours). Three larynges each were harvested from nonsmokers (cases 7, 8 and 11) and ex-smokers (Table I). Five of the other cases were active smokers and one case had an indeterminate history of tobacco use.

All of the specimens underwent amplification with human betaglobin primers. HPV 11 was isolated from three cases, no other viral subtypes were found.

Discussion

The 33 per cent prevalence rate of HPV 11 found in the present study is not significantly different from that shown for sites of invasive squamous cell carcinoma or adjacent histologically normal sites in the larynges studied by Morgan *et al.* (1991) (Fisher's exact test: $p>0.05$). The results are also within the 95 per cent confidence range of 17/69 per cent (calculated on the standard error of a proportion using the binomial approximation) illustrated by Bryan *et al.* (1990) for the histologically normal nasopharynx.

TABLE I
AUTOPSY DETAILS AND PCR RESULTS OF THE CASES STUDIED

Patient no.	Age (years)	Sex	Postmortem time (hours)	Smoker	HPV type
1	43	male	144	yes	
2	68	female	48	yes	
3	27	male	72	yes	11
4	54	male	48	yes	11
5	80	male	144	ex	
6	82	male	48	ex	
7	81	female	24	none	
8	68	female	48	none	
9	66	male	96	ex	
10	74	male	48	?	
11	61	male	72	none	
12	71	female	96	yes	11

HPV 18 was not demonstrated. There is however only one report of a laryngopharyngeal cancer positive for HPV 18 in the literature (Ogura *et al.*, 1991). The absence of HPV 16 in this study is different from the prevalence rate of 40 per cent found in other studies utilizing the polymerase chain reaction on invasive squamous cell carcinomas of the larynx Kiyabu *et al.*, 1989; Hoshikawa *et al.*, 1990; Morgan *et al.*, 1991) ($p<0.5$: Fisher's exact test one-sided). The failure to isolate these types in the present study supports an aetiological role for HPV 16 and possibly 18 in laryngeal cancer. The prevalence of HPV 11 found suggests that this viral type is not tumour-specific and can occur in the absence of laryngeal pathology.

The use of autopsy material precludes the sample from being representative of the general population, though it avoids the bias inherent in studies which obtain material from patients undergoing laryngeal procedures. A large scale, population-based, cross-sectional survey is the only assured method of determining the true prevalence of laryngeal HPV carriage.

Conclusion

This is the first study to confirm the viability of isolating HPV subtypes in autopsy larynges and provides evidence of HPV 11 infection in a larynx which appears normal.

References

Brandasma, J. L., Abramson, A. L. (1989) Association of papillomavirus with cancers of the head and neck. *Archives of Otolaryngology, Head and Neck Surgery* **115:** 621–625.

Bryan, R. L., Bevan, I. S., Crocker, J., Young, L. S. (1990) Detection of HPV 6 and 11 in tumours of the upper respiratory tract using the polymerase chain reaction. *Clinical Otolaryngology* **15:** 177–180.

Dekmezian, R. H., Batsakis, J. G., Goepfert, H. (1987) *In situ* hybridisation of papillomavirus DNA in head and neck squamous cell carcinoma. *Archives of Otolaryngology, Head and Neck Surgery* **113:** 819–821.

Griffin, N. R., Bevan, I. S., Lewis, F. A., Wells, M., Young, L. S. (1990) Demonstration of multiple HPV types in normal cervix and in cervical squamous cell carcinoma using the polymerase chain reaction on paraffin wax embedded material. *Journal of Clinical Pathology* **43:** 52–56.

Hoshikawa, T., Nakajima, T., Uhara, H., Gotoh, M., Shimosato, Y., Tsutsumi, K., Ono, I., Ebihara, S. (1990) Detection of human papillomavirus DNA in laryngeal squamous cell carcinomas by polymerase chain reaction. *Laryngoscope* **100:** 647–650.

Kashima, H., Mounts, P., Kuhajda, F., Loury, M. (1986) Demonstration of human papillomavirus capsid antigen in carcinoma *in situ* of the larynx. *Annals of Otology, Rhinology and Laryngology* **95:** 603–607.

Kiyabu, M. T., Shibata, D., Arnheim, N., Martin, W. J., Fitzgibbons, P. L. (1989) Detection of human papillomavirus in formalin-fixed invasive squamous carcinomas using the polymerase chain reaction. *American Journal of Surgical Pathology* **13:** 221–224.

Morgan, D. W., Abdullah, V., Quiney, R., Myint, S. (1991) Human papilloma virus and carcinoma of the laryngopharynx. *Journal of Laryngology and Otology* **105:** 288–290.

Ogura, H., Watanabe, S., Fukushima, K., Masuda, Y., Fujiwara, T., Yabe, Y. (1991) Presence of human papillomavirus type 18 DNA in a pharyngeal and a laryngeal carcinoma. *Japanese Journal of Cancer Research* **82:** 1184–1186.

Terry, R. M., Lewis, F. A., Griffiths, S., Wells, M., Bird, C. C. (1987) demonstration of human papillomavirus types 6 and 11 in juvenile laryngeal papillomatosis by *in situ* hybridisation. *Journal of Pathology* **153:** 245–248.

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