Comparative in situ hybridisation study of juvenile laryngeal papillomatosis in Papua New Guinea and Australia

R G Wright, D P Murthy, A C Gupta, N Cox, R A Cooke

Abstract

A comparative study of cases of juvenile laryngeal papillomatosis from Papua New Guinea (n = 3) and Brisbane, Australia (n = 9) was carried out. In situ hybridisation reactions for human papillomavirus (HPV) types 6 and 11 occurred in 11 cases. All three cases from Papua New Guinea and eight from Australia gave positive signals. A negative reaction was observed in one Australian case. The intensity of the reaction was strong in seven cases, moderate in one, and weak in three. An equivocal reaction was also noted with probes for types 16 and 18, and types 31, 33, and 35 in two cases from Australia and one from Papua New Guinea.

It is concluded that as similar staining patterns and intensities occurred in cases from both areas, the aetiology is the same. The equivocal reactions noted in three cases were probably due to cross hybridisation rather than multiple infection.

Juvenile laryngeal papillomatosis is part of a spectrum of diseases characterised by recurrent papillomata of the mucous membrane of the upper respiratory and food passages. The larynx is the site most often affected, and is often the only site. Lesions are frequently multiple and often recur even after radical treatment.

The condition is manifest clinically with progressive huskiness of the voice. Stridor and acute respiratory obstruction may occur late in the disease, necessitating tracheostomy.¹ The incidence of papillomatosis is low, but its management is difficult because of its tendency for rapid recurrence.

A viral aetiology for laryngeal papillomatosis was first suggested in 1928.² Viral particles in the specimens have been shown electron microscopically.^{3,4} The presence of human papillomavirus (HPV) has been shown by immunofluorescence techniques,⁵ and HPV types 6 and 11 have been shown in papillomata by Southern blotting techniques with radioactive probes.⁶

In situ hybridisation with radioactive probes permits the identification and localisation in tissue of HPV subtypes.⁷ Recently this has also been accomplished with biotinylated probes,⁸ a rapid method suitable for routine

laboratory use. A recent large series of adult and juvenile cases showed positive reactions for HPV types 6 and 11 in about two thirds of the cases studied.⁹ Another series of juvenile cases, however, showed 100% positivity for HPV types 6 and 11.¹⁰

Juvenile laryngeal papillomatosis has been well documented in Papua New Guinea¹¹ and in Australia.¹² This study was carried out to evaluate in situ hybridisation reactions and to compare the prevalence of HPV types 6 and 11 in cases of juvenile laryngeal papillomatosis from both regions.

Methods

Paraffin wax embedded tissue from 12 cases diagnosed in Papua New Guinea (n = 3) and Brisbane, Australia (n = 9) was retrieved from the pathology files of the Department of Pathology, Faculty of Medicine, Port Moresby General Hospital, Papua New Guinea and the Department of Anatomical Pathology, Royal Brisbane Hospital. The clinical features of the 12 cases were sought and these are presented in table 1. Most of these patients were female. Hoarseness of voice was the most common symptom. Multiple recurrent lesions and lesions at multiple sites were commonly observed.

Paraffin wax sections from formalin fixed tissues were cut at 5 µm and placed on slides pretreated with 3-aminopropyltriethoxysilane. The in situ hybridisation was carried out using the HPV tissue hybridisation kit (Vira Type in situ; Life Technologies, Inc—Bethesda Research Laboratories, Gaithersburg, Maryland, USA). Biotinylated probes for HPV genotypes 6 and 11, 16 and 18 and 31, 33, and 35 were used, with one section for each probe group.

The method entails partial denaturation of DNA (heating the sections at 100°C on metal trays in an oven following proteolytic digestion and application of the probes), incubation with hybridisation mixture (at 37°C for two hours), detection of hybridisation using a streptavidin alkaline phosphatase conjugate which binds to the biotin on the probe¹³ and dephosphorylation by alkaline phosphatase of the substrate 5-bromo-4-chloro-3 indolyl-phosphate (BCIP) in the presence of nitrobluetetrazolium (NBT). The reaction results in deposition of purplish blue precipitates at the sites of probe hybridisation to HPV DNA.¹⁴ The method described is based on

Royal Women's
Hospital and
Queensland Cytology
Services
R G Wright

Department of
Pathology, Faculty of
Medicine, Boroko,
Papua New Guinea
D P Murthy

Department of
Surgery, Port
Moresby General
Hospital, Boroko,
Papua New Guinea
A C Gupta

Queensland Cytology
Services, Brisbane
N Cox

Anatomical
Pathology, Royal
Brisbane Hospital,
Brisbane
R A Cooke

Correspondence to:
Dr R G Wright, Department
of Pathology, Royal Brisbane
Hospital, Herston Road,
Brisbane Q 4029, Australia.

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Table 1 Cases of juvenile laryngeal papilloma studied by *in situ* hybridisation

Case No	At first presentation	Sex	Country	Clinical features at first presentation	Site of papillomata	Treatment	Follow up
1	3 y 6 m	F	Papua New Guinea	Progressive hoarseness of voice and dyspnoea—3 months	Uvula, right tonsillar pillar; tip of epiglottis, true and false vocal cords, anterior commissure; cricoaryte	Initial tracheostomy. Subsequent excision with cupped forceps at microlaryngoscopy	15 months; multiple recurrences
2	5 y	F	Papua New Guinea	Inability to talk clearly and respiratory stridor—3 months	False and true vocal cords	Excision with cupped forceps	3 m
3	1 y 7 m	M	Papua New Guinea	Progressively increasing dyspnoea 6 months	True and false vocal cords; anterior commissures, Trachea	Initial tracheostomy subsequent excision with forceps	6 m
4	7 y 7 m	F	Australia	Increasing respiratory distress, huskiness of voice—6 months	Vocal cords, epiglottis, pharynx	Initial tracheostomy followed later by excision with forceps	3 y
5	9 y 6 m	F	Australia	Hoarseness of voice; recurrent laryngeal papillomata since the age of 7 y	Vocal cords, anterior commissure, laryngeal surface of epiglottis	Diathermy excision	9 y 11 m
6	9 m	F	Australia	Difficulty in breathing—3 weeks, stridor; distress	True and false vocal cords	Diathermy excision	1 y 8 m
7	2 y	F	Australia	Vomiting, respiratory stridor, hoarseness of voice 6 months, noisy breathing and difficulty—since the age of 5 months	Right and left vocal cords, posterior pharyngeal wall in hypopharynx	Multiple excisions	5 m
8	4 y 6 m	F	Australia	Hoarseness of voice	False cords (right and left)	Multiple excisions	1 y 7 m
9	1 y 6 m	F	Australia	Huskiness of voice—1 month	Vocal cords (right and left) Inferior surface of epiglottis	Multiple excisions	3 y 5 m
10	4 y 4 m	F	Australia	Hoarseness of voice	Both vocal cords, posterior pharyngeal wall, posterior oesophageal wall	Multiple excisions	1 y 2 m
11	11 y 8 m	M	Australia	Gradual onset of hoarseness of voice	Both vocal cords	Excision	5 y
12	6 y 6 m	M	Australia	Hoarseness of voice—2 years	Vocal cords, pharyngeal surface of arytenoid, anterior commissure	Excision	1 y 7 m

colorimetric *in situ* hybridisation techniques.^{8 15 16} A positive control probe for human genomic DNA and a negative control probe for unrelated DNA sequences were used with each section tested.

The slides were examined by three of the authors (RGW, DPM, and NC) and the intensity of the positive signal was graded from weak (+), moderate (++), to strong (+++). Any equivocal staining was regarded as negative. The tissue location of the positive signal was noted in each case. The slides were examined without prior knowledge of the origin of the tissue.

Table 2 Results of *in situ* hybridisation

Case No	Country	Reaction with probe for HPV types 6 and 11
1	Papua New Guinea	+
2		+
3		+++
4	Australia	+++
5		+++
6		++
7		+++
8		+++
9		+
10		+++
11		—
12		+++

Results

The reaction with the probe for HPV types 6 and 11 was found in 11 of the 12 cases studied (table 2). All three cases from Papua New Guinea and eight of the nine Australian cases gave positive signals. A negative reaction was observed in one Australian case. The reaction with the probe for HPV types 6 and 11 was present in the nuclei of the superficial epithelium in the positive cases (figure). No difference in the pattern of reaction of the probes was observed between the two groups of cases from the two regions. The intensity of the reaction was strong in seven cases, moderate in one case, and weak in three. An equivocal reaction was also noted with probes for types 16 and 18, and types 31, 33 and 35 in three cases, two from Australia and one from Papua New Guinea.

Discussion

The results of the *in situ* hybridisation tests for HPV types 6 and 11 show similar patterns and intensity of staining in cases from both areas. The results obtained are in keeping with those reported from other areas.^{9 10} Quiney has speculated that variation in hybridisation reactions may be the result of variation in fixation of tissue rather than

Positive staining for HPV types 6 and 11 in superficial cell nuclei of laryngeal papilloma.

attributable to geographical location.⁹ The results of this study support this hypothesis as there was no significant difference between the cases from the two regions investigated. From the results obtained juvenile laryngeal papillomatosis seems to have a similar aetiology in Papua New Guinea and Australia.

The equivocal reactions obtained with the probes for HPV types 16 and 18, and types 31, 33, and 35 have not been previously reported. These can probably be explained by the nature of the detection. The Vira Type assay uses low stringency conditions to achieve higher sensitivity. This has been reviewed by Lorincz.¹⁷

The weak positive reaction with the probes for HPV types 16 and 18 and types 31, 33, and 35 was seen in three cases which were strongly positive for HPV types 6 and 11. As the stringency conditions allow a degree of cross hybridisation between closely related HPV types, these weak reactions were assumed to

be due to this cross reactivity rather than to the presence of a multiple infection. We consider that caution in the interpretation of these reactions should prevail and support the methods of interpretation outlined by Quiney *et al.*⁹

- 1 Strong MS. Recurrent respiratory papillomatosis. *Scott Brown's Otolaryngology*. Vol 6. 5th edn. London: Butterworths, 1987:466-70.
- 2 Ullman EV. On the aetiology of laryngeal papilloma. *Acta Otolaryngol* 1928;5:317-38.
- 3 Incze JS, Lui PS, Strong MS, Vaughan CW, Clemente MP. The morphology of human papillomas of the upper respiratory tract. *Cancer* 1977;39:1634-46.
- 4 Boyle WF, Riggs JL, Oshiro LS, Lennette EH. Electron microscopic identification of papova virus in laryngeal papillomata. *Laryngoscope* 1978;83:1102-8.
- 5 Steinberg B, Topp WC, Schneider O, Abramson A. Laryngeal papilloma virus infection during clinical remission. *N Engl J Med* 1983;308:1262-4.
- 6 Gissman L, Wolnick L, Ikenburg H, *et al.* Human papilloma virus types 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical cancers. *Proc Natl Acad Sci USA* 1983;80:560-3.
- 7 Gupta J, Gendelman HE, Naghasfar Z, *et al.* Specific identification of human papilloma virus type in cervical smears and paraffin sections by in situ hybridization with radioactive probes. *Int J Gynecol Pathol* 1985;4:211-8.
- 8 Lewis FA, Griffiths S, Dunicliff R, Wells M, Dudding N, Bird CC. Sensitive in situ hybridization technique using biotin-streptavidin-polyalkaline phosphatase. *J Clin Pathol* 1987;16:36.
- 9 Quiney RE, Wells M, Lewis FA, Terry RM, Michaels L, Croft CB. Laryngeal papillomatosis: Correlation between severity of disease and presence of HPV 6 and 11 detected by in situ hybridisation. *J Clin Pathol* 1989;42:694-8.
- 10 Terry RM, Lewis FA, Robertson S, Blythe D, Wells M. Juvenile and adult laryngeal papillomata; classification by in situ hybridization for human papillomavirus. *Clin Otol* 1989;14:135-40.
- 11 Murthy DP, Gupta AC, Igo JD, Maku J, Mary J. Juvenile laryngeal papillomatosis: Report of cases from Papua New Guinea. *Ann Trop Paediatr* 1990;10:117-22.
- 12 Benjamin BN, Gatenby PA, Kitchen R, Harrison H, Cameron K, Basten A. Alpha interferon (Wellferon) as an adjunct to standard surgical therapy in the management of recurrent respiratory papillomatosis. *Ann Otol Rhinol Laryngol* 1988;97:376-80.
- 13 Bayer EA, Wilchek M. The use of avidin-biotin complex as a tool in molecular biology. *Method Biochem Anal* 1979;26:1-45.
- 14 McGadey J. A tetrazolium method for nonspecific alkaline phosphatase. *Histochemie* 1970;23:180-4.
- 15 Brigati DJ, Myerson D, Leary JJ, *et al.* Detection of viral genomes in cultured cells and paraffin embedded tissue sections using biotin-labelled hybridization probes. *Virology* 1983;126:32-50.
- 16 Gody WW, Cheng L, Lewin KJ. In situ viral DNA hybridization in diagnostic surgical pathology. *Human Pathol* 1987;18:535-43.
- 17 Lorincz AT. Detection of Human Papillomavirus by nucleic acid hybridization. *Obstet Gynecol Clin North Am* 1987;14:451-69.