VIROLOGY

A high prevalence of human papillomavirus DNA in recurrent nasal papillomas

H. OGURA, K. FUKUSHIMA* and S. WATANABE*

Department of Molecular Genetics, Institute of Cellular and Molecular Biology and *Department of Otorhinolaryngology, Okayama University Medical School, Okayama, Japan

The prevalence of human papillomavirus (HPV) DNA in nasal papillomas was examined by polymerase chain reaction (PCR) and Southern blot hybridisation. HPV 6 DNA in one case, HPV 57 DNA in one case and HPV 16 DNA in three cases were detected amongst 12 cases of nasal papillomas that comprised three cases of fungiform exophytic papillomas and nine cases of inverted papillomas. Five cases (two exophytic and three inverted papillomas) were recurrent and four (80.0%) of these were HPV DNA-positive. The remaining seven cases were non-recurrent and only one (14.3%) was HPV DNA-positive. This difference in HPV DNA detection rates between recurrent and non-recurrent nasal papillomas was statistically significant.

Introduction

Nasal papillomas are rare tumors of the nasal cavity and paranasal sinuses. They occur either as fungiform papillomas or inverted papillomas. Fungiform papillomas arise most commonly from the anterior nasal septum and are exophytic. Inverted papillomas arise most commonly from the lateral nasal walls. Their recurrence rates vary from 28% to 67% even after adequate removal [1]. The rate of malignant conversion ranges from 3% to 32% [1].

Nasal papillomas have been shown to contain human papillomavirus (HPV) structural proteins by immunohistochemistry [2]. Furthermore, HPV DNA has been demonstrated in the lesion by molecular hybridisation and polymerase chain reaction (PCR) [1]. So far, HPV types 6/11 [3–13], 57 [14–16], and 16/18 (in cancer associated cases) [4, 5, 7–9, 11, 13] have been detected in nasal papillomas. The frequency of HPV DNA detection in nasal papillomas has been variable (6–89%) according to the detection method used, the particular DNA probes or primers, and the patients examined [1, 3–13, 15].

This study examined the prevalence of HPV DNA types 6, 11, 16, 18 and 57 in nasal papillomas in Okayama district in Japan by PCR and Southern blot hybridisation methods.

Materials and methods

HPV DNA probes and HPV type-specific primers

Cloned DNA of HPV types 6b, 16, 18 and 57 were obtained from Drs E-M. de Villiers and H. zur Hausen, Heidelberg, Germany and that of HPV type 58 from Dr T. Matsukura, Tokyo, Japan. DNA of types 2a, 5b and 11 were cloned in this laboratory. The HPV type-specific primers for PCR are shown in Table 1. The sequence of PCR primers for HPV types 6 and 11 were the same as those reported by Melchers *et al.* [17], for HPV types 16 and 18 by Shimada *et al.* [18] and for HPV type 57 by Wu *et al.* [15]. The oligonucleotides were synthesised on an Applied Biosystems DNA synthesiser (Foster City, CA, USA).

Papilloma specimens

These were obtained from 12 patients treated operatively for papillomas of the nasal cavities and paranasal sinuses; the patients (five females and seven males) were 31–73 years of age (Table 2). A sample of each papilloma was kept frozen at -70°C until DNA extraction and the remaining portion was fixed and processed for histological examination.

Extraction of DNA

DNA was isolated from tissue homogenates by proteinase K digestion; deproteinisation with phenol, phenol:chloroform (1:1), and chloroform; precipitation with ethanol; resuspension and treatment with ribonuclease A; re-extraction with chloroform; and reprecipitation with ethanol as described previously [19].

Received 11 Sept. 1995; revised version accepted 23 Jan. 1996.

Corresponding author: Dr H. Ogura.

Table 1. Primers used for HPV DNA detection by PCR

Virus type and region	Primer sequences used	Amplimer length (bp)
HPV 6/E5	5'-TAGTGGGCCTATGGCTCGTC-3' 5'-TCCATTAGCCTCCACGGGTG-3'	280
HPV 11/L1	5'-GGAATACATGCGGCCATGTG-3' 5'-CGAGCAGACGTCCTCGTCG-3'	360
HPV 16/E6	5'-AAGGGCGTAACCGAAATCGGT-3' 5'-GTTTGCAGCTCTGTGCATA-3'	140
HPV 18/E6	5'-AAGGGCGTAACCGAAATCGGT-3' 5'-GTGTTTCAGTTCCTGTCACA-3'	140
HPV 57/E2	5'-CATGATATCTCGGCTGGG-3' 5'-AGGCACAGCGTCAGAGTCAC-3'	526

Table 2. Prevalence of HPV DNA in nasal papillomas

Case no.	Sex	Age	Recurrence no.	Growth	HPV type
1	M	58	3	Inverted	57
2	F	38	2	Inverted	16
3	M	49	2	Exophytic	16
4	M	60	1	Exophytic	6
5	F	31	1	Inverted	—
6	F	31	0	Exophytic	—
7	M	40	0	Inverted	—
8	F	50	0	Inverted	—
9	M	51	0	Inverted	16
10	M	53	0	Inverted	—
11	F	54	0	Inverted	—
12	M	73	0	Inverted	—

M, male; F, female; —, not detected.

Detection of HPV DNA by polymerase chain reaction

Amplification of DNA by PCR was done in 50- μ l reaction volumes containing 0.5 μ g of template DNA in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 μ M of each primer, 200 μ M of each deoxyribonucleotide triphosphate, gelatin 0.01% and *Taq* polymerase (Perkin Elmer Cetus, Norwalk, CT, USA) 1.25 unit. The reaction was performed on a DNA Thermal Cycler (Perkin Elmer Cetus) for 30 cycles of 1 min at 94°C,

2 min at 55°C and 3 min at 72°C [20]. Ten μ l of the PCR products were electrophoresed through an agarose 3% gel and visualised by ultraviolet irradiation after staining with ethidium bromide. As positive controls for PCR, DNA isolated from HPV type 6- and 11-positive laryngeal papillomas [21], type 16-positive CaSki cells, type 18-positive HeLa cells and cloned HPV 57 DNA were used.

DNA labelling and Southern blot hybridisation

The HPV DNA separated from each vector was labelled with digoxigenin-deoxyuridine triphosphate with the kit prepared by Boehringer Mannheim, Mannheim, Germany. Restriction endonucleases were purchased from Takara-Shuzo Co., Kyoto, Japan. Digestion with these enzymes was performed according to the methods indicated by the manufacturer. Electrophoresis of DNA (2–5 μ g) was done after restriction endonuclease digestion in agarose 0.7% gel in TEA-NaCl buffer (50 mM Tris-HCl, pH 8.0, containing 20 mM sodium acetate, 2 mM Na₂EDTA and 18 mM NaCl). After agarose gel electrophoresis, DNA was transferred to Nytran membranes (Schleicher and Schuell, Dassel, Germany) by Southern blotting. After hybridisation under stringent conditions in formamide

Fig. 1. Histology of nasal papillomas: **a**, a fungiform exophytic papilloma (case 2); **b**, an inverted papilloma (case 5) showing inverted growth into underlying stroma. (H & E, $\times 150$).

50% at 42°C overnight followed by washing at 68°C, or under non-stringent conditions in formamide 20% at 42°C overnight followed by washing at 48°C, hybrids were detected by enzyme-linked immunoassay as described previously [10].

Statistical analysis

The detection rates of HPV were analysed statistically by Fisher's exact probability test. A level of $p < 0.05$ was chosen to reflect statistical significance.

Results

Histological study

Of the 12 cases of nasal papillomas examined (Table 2) three were fungiform exophytic papillomas (Fig. 1a), two of which were recurrent, and nine were inverted papillomas (Fig. 1b), three of which were recurrent. None showed evidence of malignancy.

Detection of HPV DNA by PCR

With HPV 6-specific primers, an amplified DNA band of 280 bp corresponding to that detected in positive control DNA from a laryngeal papilloma containing HPV type 6 (Fig. 2A, lane 13) was found only in DNA from case 2 (Fig. 2A, lane 2). The HPV 11-specific primers did not produce an amplicon of 360 bp with any of the samples but did with the positive control DNA from a laryngeal papilloma containing HPV type 11 (Fig. 2B, lane 13). DNA from case 5 produced an amplicon of 526 bp with HPV 57-specific primers (Fig. 2C, lane 5) corresponding to that detected from control HPV 57 DNA. PCR amplification of DNA from cases 1, 4 and 9 with HPV 16-specific primers produced an amplicon of 140 bp (Fig. 2D, lanes 1, 4 and 9) which corresponded to that produced from the positive control CaSki cell DNA (Fig. 2D, lane 13). The HPV 18-specific primers did not produce an amplicon with any of the samples but did so with the positive control HeLa cell DNA (data not shown).

Overall, five (41.7%) of the 12 cases of nasal papilloma were HPV DNA-positive (Table 2). Among them, four (80.0%) of the five recurrent cases (cases 1–5) were HPV DNA-positive whereas only one (14.3%) of seven non-recurrent cases (cases 6–12) was HPV DNA-positive. The detection rate of HPV DNA in recurrent cases analysed by Fisher's exact probability test was significantly higher than that in non-recurrent cases ($p = 0.045$).

Detection of HPV DNA by Southern blot hybridisation

DNA from case 2 hybridised under stringent conditions with the HPV 6 DNA probe after digestion with *Bam*HI, *Hinc*II and *Pst*I (Fig. 3, lanes 1, 2 and 3).

Fig. 2. Detection of HPV DNA by PCR. **M**, size marker (phix 127 DNA digested with *Hae*III). Lanes **1–12** correspond to DNA from papillomas from cases 1–12 in Table 2. Lane **13** is the positive control for: **A**, PCR products with HPV 6-specific primers; **B**, PCR products with HPV 11-specific primers; **C**, PCR products with HPV 57-specific primers; **D**, PCR products with HPV 16-specific primers.

Similarly, DNA from case 5 hybridised with the HPV 57 DNA probe after digestion by *Eco*RI, *Hinc*II and *Pst*I (Fig. 3, lanes 4, 5 and 6). DNA from case 1, 4 and 9 did not show hybridisation signals after probing with the HPV 16 DNA (data not shown). Attempts to detect possible HPV types in the nasal papillomas with mixed probes of HPV types 2a, 5b, 6, 11, 16, 18, 57 and 58 under non-stringent conditions failed (data not shown).

Discussion

HPV has been identified in various benign papillary lesions of the upper respiratory and alimentary tracts [1]. A clinical tendency for the lesion to recur even

Fig. 3. Hybridisation pattern of HPV DNA after digestion with restriction endonucleases. Lanes 1–3: DNA from case 2 hybridised with the HPV 6b DNA probe. Digestion with: lane 1, *Bam*HI; 2, *Hinc*II; 3, *Pst*I. Lanes 4–6: DNA from case 5 hybridised with the HPV 57 DNA probe. Digestion with: lane 4, *Eco*RI; 5, *Hinc*II; 6, *Pst*I.

after adequate removal suggests the possibility of papillomas being induced by HPV.

Laryngeal papillomas are classified clinically into four groups: juvenile multiple, juvenile single, adult multiple and adult single [22]. HPV type 6/11 has been detected in almost 100% of juvenile and adult multiple laryngeal papillomas, strongly suggesting a viral aetiology for these benign tumors [23–25].

Conversely, the reported prevalence of HPV in fungiform exophytic and inverted nasal papillomas has varied [1, 3–13, 15]. The methods and techniques used for detection of HPV as well as the patients' geographic location seem to influence the results. As the detection rates of HPV types 6/11 have been relatively high in fungiform exophytic papillomas (69–75%) and low in inverted papillomas (6–15%) [3, 13], Buchwald *et al.* proposed that HPV 6/11 may be involved solely in the pathogenesis of fungiform exophytic papillomas [13].

The present study examined the prevalence of HPV DNA types 16, 18 and 57 as well as types 6 and 11 in nasal papillomas. HPV 6 DNA was detected in one case, HPV 57 DNA in one case and HPV 16 DNA in three cases. HPV 16 DNA was detected only by the PCR and not by Southern blot hybridisation. The sensitivity of HPV 16 DNA detection in the system was the endpoint dilution of 0.01 pg for the PCR and 10 pg for Southern blot hybridisation [19]. This difference in sensitivity seems to explain the detection of HPV 16 DNA by PCR but not by Southern blot hybridisation. HPV DNA was detected in two (66.7%) of three fungiform exophytic papillomas and three (33.3%) of nine inverted papillomas, supporting the

proposal of Buchwald *et al.* [13]. However, the present results can be interpreted in another way. If the papillomas are classified into recurrent and non-recurrent types as for laryngeal papillomas [22], HPV DNA was detected in four (80.0%) of five recurrent but in only one (14.3%) of seven non-current nasal papillomas, significantly higher for the recurrent type. Weber *et al.* [6] reported the detection of HPV DNA types 6/11 in 16 (76%) of 21 inverted papillomas. Among them, all of seven recurrent cases contained HPV DNA. Kleini *et al.* [7] also reported that all four cases of recurrent sinonasal papillomas yielded HPV DNA types 11/16, and Fu *et al.* [12] reported all three recurrent fungiform nasal papillomas studied had HPV DNA types 6/11. Conversely, HPV was not detected in normal nasal mucosa [13]. This suggests that not only fungiform exophytic nasal papillomas but also recurrent inverted papillomas may be induced by HPV. The cause of non-recurrent nasal papillomas remains unknown.

Although all the 12 nasal papillomas examined in this study had no evidence of cancerous change, the tendency for malignant transformation of nasal papillomas [1], and especially nasal papillomas with HPV 16 DNA [7], has been reported. Therefore, careful follow-up of patients with nasal papillomas containing HPV 16 DNA would seem to be important.

References

1. Chang F, Wang L, Syrjanen S, Syrjanen K. Human papillomavirus infections in the respiratory tract. *Am J Otolaryngol* 1992; 13: 210–225.
2. Syrjanen KJ, Pyrhonen S, Syrjanen SM. Evidence suggesting human papillomavirus (HPV) etiology for the squamous cell papilloma of the paranasal sinus. *Arch Geschwulstforsch* 1983;

- 53: 77-82.
3. Brandsma J, Abramson A, Sciubba J, Shah K, Barrezueta N, Galli R. Papillomavirus infection of the nose. *Cancer Cells* 1987; **5**: 301-308.
4. Syrjanen S, Happonen R-P, Virolainen E, Siivonen L, Syrjanen K. Detection of human papillomavirus (HPV) structural antigens and DNA types in inverted papillomas and squamous cell carcinomas of the nasal cavities and paranasal sinuses. *Acta Otolaryngol (Stockh)* 1987; **104**: 334-341.
5. Raspler DS, Jahn A, Pater A, Pater MM. Isolation and characterization of papillomavirus DNA from nasal inverting (schneiderian) papillomas. *Ann Otol Rhinol Laryngol* 1987; **96**: 170-173.
6. Weber RS, Shillitoe EJ, Robbins KT *et al.* Prevalence of human papillomavirus in inverted nasal papillomas. *Arch Otolaryngol Head Neck Surg* 1988; **114**: 23-26.
7. Klemi PJ, Joensuu H, Siivonen L, Virolainen E, Syrjanen S, Syrjanen K. Association of DNA aneuploidy with human papillomavirus-induced malignant transformation of sinonasal transitional papillomas. *Otolaryngol Head Neck Surg* 1989; **100**: 563-567.
8. Brandwein M, Steinberg B, Thung S, Biller H, Dileo T, Galli R. Human papillomavirus 6/11 and 16/18 in Schneiderian inverted papillomas. In situ hybridization with human papillomavirus RNA probes. *Cancer* 1989; **63**: 1708-1713.
9. Furuta Y, Shinohara T, Sano K *et al.* Molecular pathologic study of human papillomavirus infection in inverted papilloma and squamous cell carcinoma of the nasal cavities and paranasal sinuses. *Laryngoscope* 1991; **101**: 79-85.
10. Ogura H, Kawakami T, Fujiwara T *et al.* Detection of human papillomavirus type 6f genome in nasal papillomatosis. *Acta Otolaryngol (Stockh)* 1992; **112**: 115-119.
11. Kashima HK, Kessis T, Hruban RH, Wu TC, Zinreich SJ, Shah KV. Human papillomavirus in sinonasal papillomas and squamous cell carcinoma. *Laryngoscope* 1992; **102**: 973-976.
12. Fu YS, Hoover L, Franklin M, Cheng L, Stoler MH. Human papillomavirus identified by nucleic acid hybridization in concomitant nasal and genital papillomas. *Laryngoscope* 1992; **102**: 1014-1019.
13. Buchwald C, Franzmann M-B, Jacobsen GK, Lindeberg H. Human papillomavirus (HPV) in sinonasal papillomas: a study of 78 cases using in situ hybridization and polymerase chain reaction. *Laryngoscope* 1995; **105**: 66-71.
14. de Villiers E-M, Hirsch-Behnam A, von Knebel-Doeberitz C, Neumann C, zur Hausen H. Two newly identified human papillomavirus types (HPV 40 and 57) isolated from mucosal lesions. *Virology* 1989; **171**: 248-253.
15. Wu T-C, Trujillo JM, Kashima HK, Mounts P. Association of human papillomavirus with nasal neoplasia. *Lancet* 1993; **341**: 522-524.
16. Ogura H, Fujiwara T, K Hamaya, R Saito. Detection of human papillomavirus type 57 in a case of inverted nasal papillomatosis in Japan. *Eur Arch Otorhinolaryngol* (in press).
17. Melchers W, van den Brule A, Walboomers J *et al.* Increased detection rate of human papillomavirus in cervical scrapes by the polymerase chain reaction as compared to modified FISH and Southern-blot analysis. *J Med Virol* 1989; **27**: 329-335.
18. Shimada M, Fukushima M, Mukai H, Kato I, Nishikawa A, Fujinaga K. Amplification and specific detection of transforming gene region of human papillomavirus 16, 18 and 33 in cervical carcinoma by means of the polymerase chain reaction. *Jpn J Cancer Res* 1990; **81**: 1-5.
19. Ogura H, Watanabe S, Fukushima K, Masuda Y, Fujiwara T, Yabe Y. Human papillomavirus DNA in squamous cell carcinoma of the respiratory and upper digestive tracts. *Jpn J Clin Oncol* 1993; **23**: 221-225.
20. Ogura H, Watanabe S, Fukushima K, Masuda Y, Fujiwara T, Yabe Y. Presence of human papillomavirus type 18 DNA in a pharyngeal and a laryngeal carcinoma. *Jpn J Cancer Res* 1991; **82**: 1184-1186.
21. Watanabe S, Naito Y, Kawakami T *et al.* Human papillomavirus types 6 and 11 in laryngeal papillomas. *Larynx Jpn* 1990; **2**: 165-170.
22. Lindeberg H, Oster S, Oxlund I, Elbrond O. Laryngeal papillomas: classification and course. *Clin Otolaryngol* 1986; **11**: 423-429.
23. Mounts P, Shah KV, Kashima H. Viral etiology of juvenile- and adult-onset squamous papilloma of the larynx. *Proc Natl Acad Sci USA* 1982; **79**: 5425-5429.
24. Corbitt G, Zarod AP, Arrand JR, Longson M, Farrington WT. Human papillomavirus (HPV) genotypes associated with laryngeal papilloma. *J Clin Pathol* 1988; **41**: 284-288.
25. Tsutsumi K, Nakajima T, Gotoh M *et al.* In situ hybridization and immunohistochemical study of human papillomavirus infection in adult laryngeal papillomas. *Laryngoscope* 1989; **99**: 80-85.