Jpn. J. Cancer Res. 85, 699–703, July 1994

Detection and Cloning of Human Papillomavirus DNA Associated with Recurrent Respiratory Papillomatosis in Thailand

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Recurrent respiratory papillomatosis (RRP) is highly prevalent in Thailand. In this study, we examined the presence of human papillomavirus (HPV) DNA in 25 RRP patients in Thailand by means of dot blot analysis and/or polymerase chain reaction. Eighty-four percent (21/25) of cases and 4% (1/25) of cases were positive for HPV-11 DNA and HPV-6 DNA, respectively. Three cases (3/25) were negative for all of the examined HPV types. No cases were positive for HPV-16 or 18. Furthermore, we isolated the recombinant HPV-11 DNA clone from a genomic library constructed with the DNA of RRP tissue. The restriction map of the cloned HPV DNA was identical with the map of known HPV-11 DNA. These results suggest at least that no specific HPV type or subtype is likely to be associated with RRP in Thailand.

Key words: Recurrent respiratory papillomatosis — Thailand — Human papillomavirus — Polymerase chain reaction — Molecular cloning

Recurrent respiratory papillomatosis (RRP)[1,2] is a histologically benign tumor, but it presents a serious clinical problem since repeated surgical operations, tracheotomy and endoscopic excision are sometimes necessary to maintain an open airway.[3,4] RRP is one of the most common larynx tumors in children worldwide.[1] Incidences of RRP per 100,000 children aged 0–14 years are 0.7 in Denmark,[5,6] 0.6 in the USA[7] and 0.1 in Japan.[8] On the other hand, the incidence is 2.8 in Thailand, being 5–10 times higher than those in other countries, as reported previously.[8,9] It is known that human papillomavirus (HPV)-6 and HPV-11 are most frequently found in RRP,[10] but there has been no report about HPV types associated with RRP in Thailand.

In order to elucidate the viral factor involved in the high prevalence of RRP in Thailand, we examined the types of HPV associated with RRP in 25 cases by dot blot analysis and/or polymerase chain reaction (PCR). Furthermore we compared the restriction map and the partial sequence of cloned HPV-11 DNA from the RRP tissue of a Thai patient with those of the known HPV-11 DNA.

MATERIALS AND METHODS

Materials and DNA isolation Twenty-five patients (16 males and 9 females) with RRP, who were admitted to Rajvithi Hospital and Siriraj Hospital, Bangkok, Thailand, were the subjects of this study. The average age of patients was 3.3 years old and ranged from 2 months to 9 years old. Biopsy specimens were collected from the patients at the time of endoscopic operations and stored at −80°C until DNA isolation. DNA was isolated by proteinase K treatment and phenol/chloroform extraction as described.[11]

Dot blot hybridization Five μg aliquots of DNA were serially diluted and directly spotted onto nitrocellulose membrane. Cloned HPV-6, 11, 16 and 18 DNAs used for probes were provided by Dr. zur Hausen through the Japanese Cancer Research Resources Bank.[12–15] HPV DNAs were radiolabeled with ^{32}P with a multiprime labeling kit (Amersham, Buckinghamshire, UK). The DNA blots were hybridized with each probe at 42°C in 50% formamide overnight, followed by washing under a highly stringent condition.[16] The washed membrane was dried and exposed to Kodak X-ray film (XAR-5) at −80°C overnight with intensifying screens. Titration of the cloned HPV DNAs revealed that each probe could detect as little as 10 pg of the corresponding HPV DNA.

PCR Two sets of specific primers for E6 regions of HPV-6 and 11 were designed as described by Young *et al.*[17] The sequences of the primers are as follows: 5′ primer of HPV-6b; 5′-CCTAAAGGTCCTGTTTCGAG-3′ (nt 251–270),[18] 3′ primer of HPV-6b; 5′-ACAGGTAGCACCGAATTAGC-3′ (nt 401–420), 5′ primer of HPV-11; 5′-CGCAGAGATATATGCATATG-3′ (nt 221–240),[19]

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3′ primer of HPV-11; 5′-AGTTCTAAGCAACAGGC-AC-3′ (nt 291–301). These primers gave 170 bp and 90 bp amplified products, respectively. One μg of sample DNA was used for PCR, which was carried out using a GeneAmp kit (Takara, Co. Ltd., Kyoto) according to the manufacturer's instructions. Amplification of HPV DNAs was performed by 25 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s) and extension (72°C, 1 min) in an automatic thermocycler (Astek Co. Ltd., Fukuoka). The amplified products were electrophoresed on 3% NuSieve™ agarose and 1% agarose-1 and visualized with ethidium bromide. The PCR showed approximately 50 times higher sensitivity than the dot blot analysis in the titration experiments with cloned HPV DNAs.

Genomic cloning of HPV DNA A genomic library from an HPV-11-positive DNA (SR006, see Table I) was constructed in bacteriophage vector EMBL3 using the *Bam*HI site. *Bam*HI-digested SR006 DNA was ligated into the purified arms of EMBL3 with T4 DNA ligase and packaged *in vitro*. Then recombinant bacteriophage was inoculated into *E. coli* LE392. The HPV DNA-positive recombinants were screened and purified three times by the plaque hybridization technique[20] using ^{32}P-labeled HPV-11 DNA. The recombinant phage DNA was digested with *Bam*HI and analyzed by Southern blot hybridization, showing an approximately 8 kbp fragment which hybridized with HPV-11 under the hyghly stringent condition. Subsequently, the insert DNA in the HPV DNA-positive recombinant was subcloned into pUC118 at the *Bam*HI site. The established clone was named Thai papilloma genome-1 (TPG-1). *Ava*II-*Pst*I fragment (nt 33–572) and *Hin*dIII-*Xba*I fragment of TPG-1 including the E6 and L2 region, respectively, were subcloned into pUC118 and the nucleotide sequences of these regions were determined by Sanger's dideoxy method[21] using an AmpliTaq™ sequencing kit (Takara, Co. Ltd.).

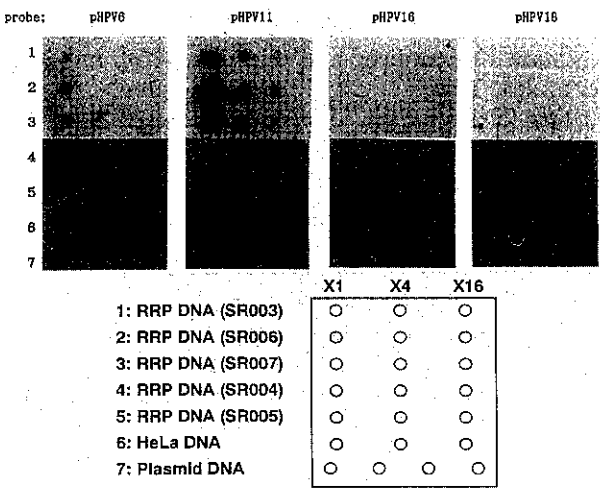

Fig. 1. Dot blot hybridization of DNA isolated from RRP tissue. The DNA was serially diluted and spotted onto nitrocellulose membrane (X1: 5 μg, X4: 5/4 μg, X16: 5/16 μg of DNA). Four patients (SR003, SR005, SR006 and SR007) were positive for HPV-11 DNA and one patient (SR004) was positive for HPV-6 DNA. Weak signals of HPV-6 for two patients (SR006 and SR007) were thought to be false-positive because sequences of HPVs 6 and 11 are highly homologous. HeLa cell and pBR322 DNA were used as controls.

RESULTS

Investigation of HPV types among RRP in Thai patients The results of dot blot hybridization and PCR analysis are indicated in Figs. 1 and 2 and Table I. Out of 25 patients, 21 cases (84%) were positive for HPV-11 DNA, one case (4%) was positive for HPV-6 DNA and three cases were negative for all of the HPV types examined. No patient was positive for HPV-16 or 18. The results of dot blot analysis and PCR were consistent. Two cases were positive for HPV-11 by PCR, but negative by dot blot analysis. This may be due to the difference in the sensitivity of the two detection methods.

Cloning of HPV DNA from RRP in Thai patients and characterization of the cloned HPV DNA Dot blot hybridization and PCR revealed that HPV-11 is the predominant type of HPV in RRP of Thai patients. We screened a genomic library constructed from the DNA of the HPV-11-positive RRP tissue (SR006) and isolated a recombinant phage clone which harbors the HPV DNA.

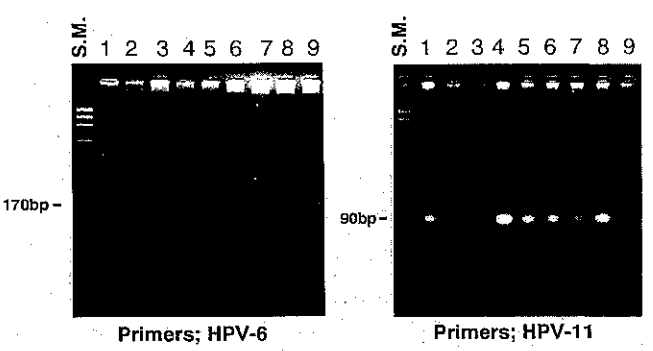

Fig. 2. PCR analysis of DNA isolated from RRP tissue. One μg of DNA was amplified with specific primers for HPV-6 HPV-11. Lane S.M. was ØX174 DNA digested with *Hae*III as a size marker. Lanes 1 to 9 were SR003, 004, 005, 006, 007, 011, 014, 015 and HeLa DNA, respectively. (Left) DNAs were amplified with HPV-6 specific primers. The specific product (170 bp in size) was seen in patient SR004 (lane 2). (Right) DNAs were amplified with HPV-11 specific primers. The specific product (90 bp in size) was seen in 7 patients (lanes 1 and 3–9).

Table I. Clinical Profiles of Patients and Detection of HPV DNA

Patient No.	Sex	Age at onset	Site of papilloma	HPV DNA	
				Dot	PCR
SR001	F	5	Glottis, Supraglottis	11	11
SR002	F	5	Glottis, Supraglottis	11	11
SR003	M	4	Glottis	11	11
SR004	F	3	Glottis, Supraglottis	6	6
SR005	M	4	Glottis, Supraglottis	11	11
SR006	F	6	Glottis, Supraglottis	11	11
SR007	M	3	Glottis, Supraglottis	11	11
SR008	M	3	Glottis, Supraglottis	– [a]	–
SR009	M	5	Glottis	11	11
SR010	F	4	Glottis, Supraglottis	–	11
SR011	F	5	Glottis, Sub & Supraglottis	11	11
SR013	M	3	Glottis	–	–
SR014	M	3	Glottis, Sub & Supraglottis	11	11
SR015	F	4	Glottis, Supraglottis	11	11
SR016	F	4	Glottis, Supraglottis	11	11
SR017	F	3	Glottis, Supraglottis	–	11
SR018	M	4	Glottis, Supraglottis	11	11
RAJ005	M	2	Glottis, Sub & Supraglottis	11	11
RAJ010	F	2	Glottis, Supraglottis	11	11
RAJ014	M	1	Glottis	11	11
RAJ017	F	1	Glottis, Sub & Supraglottis	ND [b]	11
RAJ021	M	1	Glottis, Supraglottis	ND	11
RAJ022	M	2M	Glottis	11	11
RAJ023	M	4M	Glottis, Supraglottis	ND	11
RAJ024	M	9	Glottis	ND	–

a) – : not detected.
b) ND: not determined.

Then the approximately 8 kbp insert was subcloned into pUC118 at the *Bam*HI site.

TPG-1 was digested with several restriction enzymes as shown in Fig. 3A. The approximately 3.2 kbp fragment in all three lanes (2, 3 and 4) was judged to be pUC118 DNA. The insert was found to have one restriction site of *Hin*dIII and at least four of *Pst*I, and the *Hin*dIII site was found to be located within the second-longest *Pst*I fragment. In addition, the insert DNA had at least four restriction sites of *Ava*II (lane 6), and the *Hin*dIII site was located within the third-longest *Ava*II-fragment (lane 7). The digestion patterns of TPG-1 DNA by the restriction enzymes were fully consistent with that of the published sequence of HPV 11 DNA[18] (Fig. 3B). Furthermore, we determined the partial nucleotide senquences of the *Ava*II-*Pst*I (nt 33–572) and *Hin*dIII-*Xba*I (nt 4557–5887) regions and found that these sequences of TPG-1 were fully consistent with the published sequence[19] of HPV-11 DNA.

DISCUSSION

Dot blot hybridization and PCR studies showed that HPV-11 DNA and HPV-6 DNA were positive in 84% and 4% of the Thai RRP patients, respectively. Abramson *et al.*[4] reported that HPV-11 and HPV-6 DNA were detected in 59% (10/17) and 41% (7/17) of cases of adult onset RRP, respectively. Gissmann *et al.*[10] reported that HPV 11 DNA was detected in 50% (7/14) of cases whereas no HPV 16 DNA was detected in adults. Thus, HPV-11 seems a more predominant type in Thai RRP as compared with previous reports, although Smith *et al.*[22] recently reported a predominance of HPV-11 detected by PCR among RRP patients in the USA.

There was no difference between the cloned HPV-11 DNA from RRP in Thai patients and the known HPV-11 DNA sequence[19] in the restriction map and partial sequence. Thus, at least no specific type or subtype of HPV is likely to be associated with the RRP in Thai patients. Chan *et al.*[23] examined mutation of the long control region and E5 open reading frame of HPV-16 DNAs isolated from anogenital region biopsy samples and found that HPV 16 has at least two major variants which are associated with geographical location. Although it is not clear whether there is a difference of virulency among HPV variants, we cannot exclude the possibility that a unique variant of HPV-11 associated with RRP in Thailand could contribute to the high prevalence.

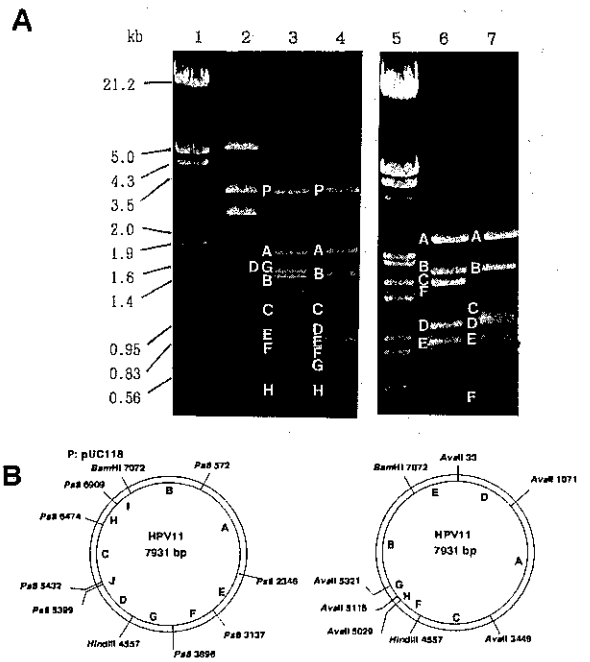

Fig. 3. Restriction mapping of HPV-11 DNA cloned from a Thai patient. (A) Lane 1; λDNA digested with *Hin*dIII and *Eco*RI. Lane 2; TPG-1 digested with *Bam*HI and *Hin*dIII. Lane 3; TPG-1 digested with *Bam*HI and *Pst*I. Lane 4; TPG-1 digested with *Bam*HI, *Pst*I and *Hin*dIII. Lane 5; λDNA digested with *Hin*dIII and *Eco*RI. Lane 6; Insert DNA digested with *Ava*II. Lane 7; Insert DNA digested with *Ava*II and *Hin*dIII. (B) The restriction map based on the published sequence of the HPV-11 DNA. The restriction pattern of cloned HPV DNA corresponded completely to that of the HPV-11 DNA sequence.

Mother-to-child transmission of HPV has been suggested in children born to mothers having a history of genital condyloma.[4, 24–26] In this study, 3 cases (12%) had been born to mothers with a history of genital condyloma (RAJ005, RAJ010 and RAJ014 in Table I). HPV DNA was detected in 15% to 28% of pregnant women with no symptom of genital condyloma.[27, 28] Thus, it is important to survey the prevalence of genital HPV infection prevailing in Thai women in order to elucidate the route of HPV infection among the RRP patients in Thailand.

This study showed that there is no major difference of viral etiology of RRP between Thailand and other countries, even though the prevalence of this disease is quite different among them. To elucidate the mechanism involved, a further epidemiological investigation including environmental and genetic factors will be necessary.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Cancer Research and Overseas Scientific Research Survey (0142006) from the Japanese Ministry of Education, Science and Culture. The authors thank Dr. Masaru Ohyama for encouragement and support of this study, Dr. Robert Fobes for comments on the manuscript and Dr. H. zur Hausen for supplying cloned HPV DNAs.

(Received January 20, 1994/Accepted March 28, 1994)

REFERENCES

1) Strong, M. S., Vaugman, C. W., Hearly, G. B., Cooperband, S. R. and Clemente, M. A. C. P. Recurrent respiratory papillomatosis: management with CO_2 laser. *Ann. Otol. Rhinol. Laryngol.*, **85**, 508–516 (1976).
2) Kashima, H. K. and Shah, K. Recurrent respiratory papillomatosis, clinical overview and management principles. *Obstet. Gynecol. Clin. North Am.*, **14**, 581–588 (1987).
3) Benjamin, B. and Parsons, D. S. Recurrent respiratory papillomatosis: a 10 year study. *J. Laryngol. Otol.*, **102**, 1022–1028 (1988).
4) Abramson, A. L., Steinberg, B. M. and Winkler, B. Laryngeal papillomatosis: clinical, histopathologic and molecular studies. *Laryngoscope*, **97**, 678–685 (1987).
5) Christensen, P. H., Jǿrgensen, K. and Grǿntved, A. Juvenile papillomatosis of the larynx. *Acta Otolaryngol. Suppl.*, **412**, 37–39 (1984).
6) Bomholt, A. Juvenile laryngeal papillomatosis. *Acta Otolaryngol.*, **105**, 367–371 (1988).
7) Mount, P. and Kashima, H. K. Association of human papillomavirus subtype and clinical course in respiratory papillomatosis. *Laryngoscope*, **94**, 28–33 (1984).
8) Ohyama, M., Antarasena, S., Sonoda, S., Yoshida, H., Oda, H., Fujiyoshi, T., Ushikai, M., Kono, M., Sorasuchart, A. and Sanikorn, P. Juvenile laryngeal papillomatosis in Thailand. *In* "Epidemiology and Prevention of Cancer," ed. R. Sasaki, and K. Aoki, pp. 53–69 (1990). University of Nagoya Press, Nagoya.
9) Antarasena, S. Clinical study of juvenile laryngeal papilloma. *Acta Otolaryngol. Suppl.*, **458**, 163–166 (1988).
10) Gissmann, L., Wolnick, L., Ikenberg, H., Koldovsky, U., Schnürch, H. G. and zur Hausen, H. Human papilloma virus type 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical cancers. *Proc. Natl. Acad. Sci. USA*, **80**, 560–563 (1983).
11) Sambrook, J., Fritsch, E. F. and Maniatis, T. "Molecular Cloning, A Laboratory Manual" (1989). Cold Spring

Harbor Laboratory Press, New York.
12) De Villiers, E. M., Gissmann, L. and zur Hausen, H. Molecular cloning of viral DNA from human genital warts. *J. Virol.*, **40**, 932–935 (1981).
13) Gissmann, L., Diehl, V., Schult-Coulon, H. J. and zur Hausen, H. Molecular cloning and characterization of human papilloma virus DNA derived from a laryngeal papilloma. *J. Virol.*, **44**, 393–400 (1982).
14) Dürst, M., Gissmann, L., Ikenberg, H. and zur Hausen, H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc. Natl. Acad. Sci. USA*, **80**, 3812–3815 (1983).
15) Boshart, M., Gissmann, L., Ikenberg, H., Kleinheitnz, A., Scheurlen, W. and zur Hausen, H. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO J.*, **3**, 1151–1157 (1984).
16) Achneider, A. Methods of identification of human papillomaviruses. *In* "Papillomaviruses and Human Disease," ed. K. Syrjänen, L. Gissmann and L. G. Koss, pp. 19–39 (1987). Springer-Verlag, Berlin.
17) Young, L. S., Bevan, I. S., Johnson, M. A., Blomfield, P. I., Broidge, T., Maitland, N. J. and Woodman, C. B. J. The polymerase chain reaction: a new epidemiological tool for investigation of cervical human papillomavirus infection. *Br. Med. J.*, **298**, 14–18 (1989).
18) Schwarz, E., Dürst, M., Demankowski, C., Lattermann, O., Zech, R., Wolfsperger, E., Suhai, S. and zur Hausen, H. DNA sequence and genomic organization of genital human papillomavirus type 6b. *EMBO J.*, **2**, 2341–2348 (1983).
19) Dartmann, K., Schwarz, E., Gissmann, L. amd zur Hausen, H. The nucleotide sequence and genome organization of human papilloma virus type 11. *Virology*, **151**, 124–130 (1986).
20) Benton, W. D. and Davis, R. W. Screening λgt recombinant clones by plaque hybridization to single plaques *in situ*. *Science*, **196**, 180–182 (1977).
21) Sanger, F., Nicklen, S. and Coulson, A. R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467 (1977).
22) Smith, E. M., Pignatari, S., Gray, G. D, Haugen, T. H. and Turek, L. P. Human papillomavirus infection in papillomas and nondiseased respiratory sites of patients with recurrent respiratory papillomatosis using the polymerase chain reaction. *Arch. Otolaryngol. Head Neck Surg.*, **119**, 554–557 (1993).
23) Chan, S. Y., Ho, L., Ong, C. K., Chow, V., Drescher, B., Dürst, M., Muelen, J., Villa, L., Luande, J., Mgaya, H. N. and Bernard, H. U. Molecular variants of human papillomavirus type 16 from four continents suggest ancient pandemic spread of the virus and its coevolution with humankind. *J. Virol.*, **66**, 2057–2066 (1992).
24) Mount, P. and Shah, K. Respiratory papillomatosis: etiological relation to genital tract papillomaviruses. *Prog. Med. Virol.*, **29**, 90–114 (1984).
25) Quick, C. A., Watts, S. L., Krzyzek, R. A and Faras, A. J. Relationship between condylomata and laryngeal papillomata. *Ann. Otol. Rhinol. Laryngol.*, **89**, 467–471 (1980).
26) Shah, K., Kashima, H. K., Polk, B. F., Shah, F., Abbey, H. and Abramson, A. Rarity of caesarean delivery in cases of juvenile laryngeal papillomatosis. *J. Obstet. Gynecol.*, **68**, 795–799 (1986).
27) Koutsky, L. A., Galloway, D. A. and Holmes, K. K. Epidemiology of genital human papillomavirus infection. *Epidemiol. Rev.*, **10**, 122–163 (1988).
28) Smith, E, M., Johnson, S. R., Cripe, T. P., Pignatari, S. S. and Turek, L. P. Perinatal vertical transmission of human papillomavirus and subsequent development of respiratory tract papillomatosis. *Ann. Otol. Rhinol. Laryngol.*, **100**, 479–483 (1991).