The Seoul Journal of Medicine
Vol. 31, No. 3: 201-205, September 1990

Detection of Human Papillomavirus DNA in Squamous Papilloma of the Larynx by In Situ Hybridization

Kyoung Chan Park, Hyang Joon Park[1], Jeong Wook Seo[2], Kwang Hyun Kim[3] and Yoo Shin Lee

Department of Dermatology, Pathology[2], and Otolaryngology[3], Seoul National University College of Medicine, Seoul 110-460, Korea
Department of Dermatology[1], College of Medicine, Inje University, Pusan 614-110, Korea

=Abstract=**In situ hybridization using digoxigenin labeled HPV(human papillomaviruses) probes was tried in order to detect the presence of HPV DNA in 27 cases of single and multiple laryngeal papilloma. There were HPV6/11 DNA signals in 17 out of 22 cases of multiple laryngeal papilloma and in all five cases of single laryngeal papilloma. The specific signals were seen focally in the nuclei of superficial epithelial cells. None of the cases was positive for HPV16/18. There was no cross hybridization between HPV6/11 and HPV16/18 under the stringent conditions used in these experiments. So it could be said that HPV6/11-related sequences were found in laryngeal papillomas.**

Key Words: *In situ hybridization, Human papillomaviruses, Laryngeal papilloma*

INTRODUCTION

Human papillomavirus(HPV) infection of the larynx is a serious and potentially life-threatening disease affecting both children and adults and can result in complete respiratory obstruction(Holinger et al., 1950). The most common age of onset is during the first year of life, and it is strongly suspected that such multiple juvenile laryngeal papilloma originates from a perinatal infection from mothers with condylomatous lesions(Hallden and Majmudar, 1986). Although there have been conflicting reports on the presence of virus particles in these tumors when examined by electron microscopy(Spoendlin and Kistler, 1987; Jahnke and Arnold, 1987), HPV6/11 DNA has been detected in adult and juvenile laryngeal papilloma by molecular biology techniques such as Southern hybridization(Mounts et al., 1982; Gissman et al., 1983; Tsutsumi et al., 1989).

This is the first experiment in Korea to detect HPV DNA in the laryngeal papilloma. We tried to detect HPV DNA using an in situ hybridization technique with a nonradioactive-labeled probe with digoxigenin.

This work was supported by a grant from Seoul National University Hospital(1990).

MATERIALS AND METHODS

Materials

Representative tissue blocks of 27 cases of laryngeal papilloma were selected following a histolo-

Table 1. The results of in situ hybridization in 27 laryngeal papilloma

Number	Age	Sex	Onset	Lesion	HPV6/11
1	55	M	40	single	+
2	81	M	75	single	+
3	16	F	11	single	+
4	55	F	50	single	+
5	59	M	48	single	+
6	68	M	57	multiple	+
7	24	M	21	multiple	+
8	15	F	5	multiple	−
9	44	M	37	multiple	+
10	47	M	31	multiple	−
11	37	M	28	multiple	+
12	62	F	57	multiple	+
13	40	M	25	multiple	+
14	45	M	41	multiple	+
15	46	M	29	multiple	+
16	34	M	32	multiple	+
17	36	F	26	multiple	−
18	53	M	50	multiple	−
19	40	M	36	multiple	+
20	59	M	48	multiple	−
21	34	M	29	multiple	+
22	62	M	60	multiple	+
23	59	M	27	multiple	+
24	29	M	23	multiple	+
25	31	M	27	multiple	+
26	46	M	34	multiple	+
27	45	M	43	multiple	+

gic review from the files of the Department of Pathology, Seoul National University Hospital. The tissue had been routinely formalin-fixed and embedded in paraffin. The clinical information is summarized in Table 1.

CaSki cell line, which is known to have sequences of HPV16 DNA(Pater and Pater, 1985), was included for the control of hybridization experiments.

Pretreatment of tissue sections

Sections 4 to 6 μm thick were placed on poly-l-lysine(Sigma)coated slides and hot-plated for several hours to ensure maximum adhesion. The sections were deparaffinized in xylene two times. The sections were hydrated through graded alcohols to distilled water as previously described(Tsutsumi *et al.*, 1989). Briefly, the slides were immersed in 0.2N HCl for 15 min, washed in 2X SSC, 5mM EDTA solution, then treated with a solution of proteinase K(100 ug/ml). The slides were washed twice in PBS containing 2 mg/ml glycine, then dehydrated through graded alcohols.

DNA probes

Human papillomavirus probes HPV6b, HPV11, HPV16, and HPV18 were obtained from Dr.zur Hausen. The probes were labeled with digoxigenin -dUTP(Behringer Mannheim) using a random primer method. Unincorporated dNTP were separated from the labeled DNA by the spun column technique on Sephadex G50(Sigma). The separated probes were mixed in a hybridization solution (5X SSC, 0.5% blocking solution 〈Behringer Mannheim〉, 0.1 % N-lauroyl sarcosine〈Na salt〉, 0.02% SDS).

In situ hybridization

A hybridization mixture(50 ul) containing a probe (HPV6/HPV11, HPV16/HPV18) was pipetted onto the prepared sections. The slides were covered with cover slides. The slides were placed on a hot plate and denatured for 5 min at 92℃ and transferred to an incubator at 60℃ in a humidified box for 18-42 hours. After hybridization, the slides were immersed in 2X SSC, and then the cover slides were removed carefully. The slides were then washed sequentially in 1X SSC at RT with agitation for 15 min, 0.1X SSC at 60℃ two times.

Detection of hybridization signal

The slides were processed using a nonradioactive DNA labeling and detection kit(Behringer Mannheim). Briefly, the slides were washed with buffer (100 mM Tris-HCl, 150 mM NaCl; pH 7.5) for 1 min. The slides were drained of excess buffer and incubated with polyclonal sheep anti-digoxigenin Fab-fragments and conjugated to alkaline phosphatase at 150 mU/ml for 30 min. Unbound antibody-conjugate was washed with buffer. The slides were then incubated for enzyme catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phos-

phate (X-phosphate) and nitroblue tetra-zolium salts(NBT).

RESULTS

The results of the analysis of HPV types are summarized in Table 1. Twenty two(81.5%) out of 27 cases in the selected series gave positive results with digoxigen-labeled HPV6/11. Caski cell line gave no signal with HPV6/11 probes. HPV DNA signals were detected in all five single papilloma and in 17 out of 22 cases of multiple laryngeal papilloma. The specific hybridization signal was seen focally in the nuclei of superficial squamous epithelial cells and correlated well with koilocytosis, which is a recognized morphological expression of HPV infection(Fig. 1,2). None of the lesions was positive for HPV16/18 except CaSki cel lines.

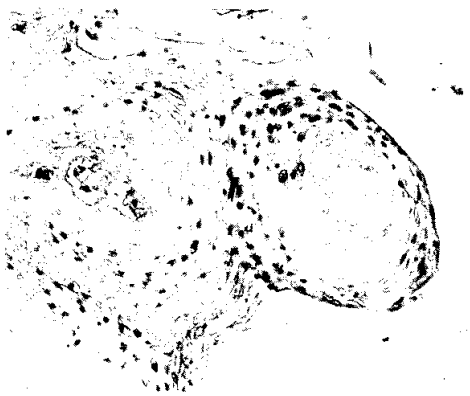

Fig. 1. Detection of HPV DNA by in situ hybridization. Specific signals accumulated on the nuclei of keratinocytes(X 100).

Fig. 2. The specific hybridization signal was seen focally in the nuclei of squamous epithelial cells and correlated well with koilocytosis(X 400).

DISCUSSION

The DNA of HPV type 6 had been cloned from human genital warts by de Villiers *et al.*(1981). Gissman *et al.*(1982) purified papilloma virus DNA from a case of juvenile onset laryngeal papilloma and determined this to be a new type as HPV11. They have shown it to be associated with genital warts and with seven of the 14 laryngeal papilloma examined(Gissman *et al.*, 1983). Since then, it is known that HPV, especially HPV6 and HPV11, plays an important role in the development of juvenile and adult multiple laryngeal papilloma.

Due to the high degree of genome sharing between these viruses, some cross reactivity was observed between HPV6 and HPV11(Corbitt *et al.*, 1988). Southern analysis with digested DNA or type specific oligonucleotide probes will be necessary for the differentiation of these viruses. In these experiments, we tried to detect the presence of HPV DNA with mixed probe of HPV6/11 or HPV16/18. Caski cell line, which is known to have HPV16 sequences, gave signal with not HPV6/11 probes but HPV16/18 probes. These results shows that there was no cross hybridization between HPV6/11 and HPV16/18 under the stringent conditions used in these experiments. Because HPV cannot be cultivated, hybridization techniques, such as Southern hybridization, dot hybridization and in situ hybridization, have been used for the detection of HPV DNA. In situ hybridization, using a radioactive probe, is a very sensitive method for investigating HPV localization in tissue. According to Tsutsumi *et al.*(1989), there were signals of HPV DNA in six out of eight cases of laryngeal papilloma examined with in situ hybridizaiton using 35S-labeled probe. From our study, there were positive signals in 81.5 % of laryngeal papilloma. These results show that in situ hybridization using a digoxigenin-labeled probe is very sensitive compared to an isotope-labeled probe. Tsutusmi *et al.*(1989) found no HPV DNA signals and/or papillomavirus genus-specific

common antigens in all samples of single laryngeal papilloma. They showed a direct association of HPV with adult multiple laryngeal papilloma, not with single papilloma. But we found HPV signals in all five single papilloma and in 77.3% of the multiple papilloma. These results show that the differentiation between single and multiple papilloma is not necessary in viral causation of laryngeal papilloma.

Recently, it has been reported that some types of HPVs may be closely related to carcinogenesis in the larynx. Bransma *et al.*(1986) demonstrated the presence of an HPV-16 related sequence in verrucous carcinoma of the larynx. Kahn *et al.* (1986) molecularly cloned a new type of HPV(HPV 30) from laryngeal carcinomas. However, our present study revealed HPV6/11 infection in single and multiple papilloma, but the study did not show HPV16/18 infection in all laryngeal papilloma, which is known to be involved in the carcinogenesis of cervix cancer and in some cases of laryngeal cancer. Further investigation is necessary to find out the relationship between HPV infection and laryngeal carcinoma.

ACKNOWLEDGEMENTS

We are grateful to H. zur Hausen for kindly providing us with cloned HPV6b, HPV11, HPV16, and HPV18. We would also like to thank Miss We Y.M. for her help in preparing samples.

REFERENCES

Bransma JL, Steinberg BM, Abramson AL. Presence of human papillomavirus type 16 related sequences in verrucous carcinoma of the larynx. Cancer Res. 1986, 46: 2185-2188

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

de Villiers EM, Gissman L, zur Hausen H. Molecular cloning of viral DNA from human genital warts. J. Virol. 1981, 40: 932-935

Gissman L, Diehl V, Schultz-Coulon HJ, zur Hausen H. Molecular cloning and characterization of human papillomavirus DNA derived from a laryngeal papilloma. J. Virol. 1982, 44: 393-400

Gissman L, Wolnik L, Ikenberg H, Koldovsky U, Schnurch HG, zur Hausen H. Human papillomavirus types 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical cancers. Proc. Natl. Acad. Sci.USA. 1983, 80: 560-563

Hallden C, Majmudar B. The relationship between juvenile laryngeal papillomatosis and maternal condyloma acuminata. J. Repro. Med. 1986, 31: 804-807

Holinger PH, Johnson KC, Anison GC. Papilloma of the larynx: Review of 109 cases with preliminary report of aureomycin therapy. Ann. Otol. Rhinol. Laryngol. 1950, 59: 547-563

Jahnke K, Arnold W. The importance of electron microscopy in research and clinical otorhinolaryngology. Anat Anz. 1987, 163: 63-78

Kahn T, Schwarz F, and zur Hausen H. Molecular cloning and characterization of the DNA of a new human papillomavirus(HPV30) from a laryngeal carcinoma. Int. J. Cancer 1986, 37: 61-65

Mounts P, Shah KV, Kashima H. Viral etiology of juvenile and adult onset squamous papilloma in the larynx. Proc. Natl. Acad. Sci. USA. 1982, 79: 5425-5429

Pater MM, Pater A. Human papillomavirus types 16 and 18 sequences in carcinoma cell lines of the cervix. Virology 1985, 145: 313-318

Spoendlin H, Kistler G. Papovavirus in human laryngeal papilloma. Ann. Otol. Rhinol. Laryngol. 1987, 218: 289-292

Tsutsumi K, Nakajima T, Gotoh M, Shimosato Y, Tsunokawa Y, Tereda M, Ebihara S, Ono I. In situ hybridization and immunohistochemical study of human papillomavirus infection in adult laryngeal papillomas. Laryngoscope 1989, 99: 80-85

= 국문초록 =

In Situ Hybridization을 이용한 후두 유두종 조직내 Human Papillomavirus DNA의 검색

서울대학교 의과대학 피부과학교실, 병리학교실[1], 이비인후과교실[2]
인제대학교 의과대학 피부과학교실[3]

박경찬 · 박향준[3] · 서정욱[1] · 김광현[2] · 이유신

후두유두종으로 진단된 27예의 환자를 병변의 수에 따라 두군으로 나누어 in situ hybridization을 시행한 결과 27예 중 22예에서 HPV6/11에 양성반응을 보였으며 hybridization 소견은 표피의 표층에 핵에 국소적으로 반응하는 소견을 보였다. 22예의 다수 병소를 보인 후두유두종에서는 17예에서, 단일 병소의 후두유두종환자에서는 5예 모두에서 HPV DNA의 염기서열이 발견되었다. 사용된 조건의 실험에서 HPV6/11과 HPV16/18은 cross hybridization을 보이지 않았으며 HPV16/18의 염기서열이 발견된 예는 없었다. 이러한 결과로 후두에 발생하는 유두종은 HPV6/11에 의해 유발되는 바이러스성 질환임을 확인할 수 있었다.