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In Situ Hybridization study on Human Papillomavirus DNA Expression in Benign and Malignant Squamous Lesions of the Esophagus

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Histologic changes suggesting HPV infection are occasionally found adjacent to squamous cell carcinoma or in squamous papilloma of the esophagus, but the relationship between HPV infection and benign and malignant squamous lesions of the esophagus is not yet clear. The aim of this study was to examine the role of HPV in squamous lesions of the esophagus. Microscopic examination with emphasis on HPV infection was done on 15 cases of squamous cell carcinoma and 26 cases of squamous papilloma. In situ hybridization technique for wide-spectrum HPV probe was performed on 35 endoscopically biopsied esophageal tissues. Among the histologic parameters suggesting HPV infection, acanthosis was the most frequent finding : 100.0% in benign and malignant esophageal lesions, and koilocytosis and intraepithelial capillary loops were the second (92.7%).: Dyskeratosis, basal cell hyperplasia and bi- or multinucleation were 52.3%, 44.0% and 34.1% in frequency, respectively. On in situ hybridization study, the HPV DNA expression rates of 10 squamous cell carcinomas with evidence of HPV infection and 15 carcinomas without evidence of HPV infection were 60.0% and 33.3%, respectively. In contrast to the carcinoma cases, only one (10.0%) of 10 squamous papillomas revealed positive signal. In conclusion, HPV infection is strongly associated with squamous cell carcinoma, but the causal relation of HPV to squamous papilloma is inconspicous.

Key Words : *Esophageal squamous papilloma, Squamous cell carcinoma, Human papillomavirus, In situ hybridization, Esophagus*

INTRODUCTION

Human papillomaviruses (HPV) are known to be intimately linked to carcinoma involving the cervix and the larynx. The specific types of HPV have different oncogenic risk, and types 16 and 18 are associated with high oncogenic risk (Syrjanen, 1986). Squamous papilloma (SP) of the esophagus is a rare lesion that has become increasingly frequent with the advent of endoscopy and which shows similar histologic findings with HPV infection in the uterine cervix. Furthermore, similar histological appearances of HPV infection in anogenital areas have been described in the lesions adjacent to esophageal squamous cell carcinoma (SCC) (Syrjanen et al.,1982). Hording et al. (1989) re-

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ported that HPV is associated with SP, and recently, many studies have been performed to prove the relationship of HPV infection and tumor occurance in the esophagus. Investigations for identification of HPV in SP and SCC by in situ hybridization (ISH) or polymerase chain reaction (PCR) have been also performed. However, the etiologic role of HPV in squamous cell lesions of the esophagus is very difficult to clarify. Furthermore, some of the previous studies reported an alternative theory, in which mucosal injury followed by regeneration played a role in the pathogenesis of esophageal SP (Quitadamo and Benson, 1988 ; Politoske, 1992 ; Odze et al., 1993 ; Carr et al., 1994).

To clarify the role of HPV infection in the development of esophageal SP and SCC, we investigated the frequency of histologic parameters suggestive of HPV infection in SP and SCC which are more common in Koreans than caucasians. We also performed *in situ* hybridization with wide-spectrum HPV DNA probe and analyzed HPV expression prevalence in three groups ; 1) SCC with evidences of HPV infection, 2) SCC without evidences of HPV infection and 3) SP.

MATERIALS AND METHODS

Case selection

Cases were from the biopsed esophageal specimens of Pusan Paik Hospital from 1991 to 1994, which were diagnosed as SP or SCC, and these were divided into three groups. The 15 cases of SCC accompanying evidences of HPV infection in adjacent areas were classified as Group A, 15 cases of SCC without evidences of HPV infection as Group B and 26 cases showing only SP as Group C.

Histological evaluation ; Histological parameters characteristic of HPV infection suggested by Syjaren et al. (1982) and Toki and Yajima (1987), such as koilocytosis, acanthosis, basal cell hyperplasia, hyperkeratosis, multinucleation, and intraepithelial capillary loops were evaluated in Group A and Group C. The findings of those were analyzed and compared with the "HPV score" system of Toki and Yajima (1987) (Table 1).

Table 1. "HPV score" system for histologic diagnosis of HPV infection

Histologic parameters	Score
Koilocytosis	4
Bi- and multinucleation	2
Dyskeratosis	1
Intraepithelial capillary loops	1
Basal cell hyperplasia	1
Acanthosis	1

HPV infection is diagnosed when 6 points or more are allocated. (cited from Toki and Yajima, 1987)

***In situ* hybridization ;** Twenty-five cases of SCC (10 of 15 Group A and 15 Group B) and 10 SPs from Group C were examined for HPV by ISH. More than three of 4-6 μ thick sections were cut in each case. Sections on organic silane pretreated slides (DAKO) were warmed in a 60℃ oven for more than 1 hour. After deparaffinization and hydration, sections were treated with ribonuclease by a Microprobe system and warmed for 1 minute at 110℃, immersed in pepsin (Research Genetics, Alabama, USA) for 3 minutes at 110℃ and in formamide for 2 minutes at 110℃. On each slide one drop of Fluorescein-Labelled wide-spectrum HPV probe (DAKO, Japan) was applied and covered with a coverslip. After a short wash with distilled water, samples were warmed for 6 minutes at 90℃ in the microprobe system and incubated at 37℃ in a humidified chamber overnight. On the following day, the coverslides were removed by soaking with TBS (0.1% triton X-100 mixed Tris-buffered saline). The hybridization procedure was finished by washing in sodium chloride sodium citrate (ssc) solution under stringent conditions (1×ssc, 30 minutes in 48℃) and TBS solution for 5 minutes. For detection, slides were kept for 20 minutes in a light-blocking box. Anti-FITC antibodies were used and this was followed by three, five minute washings in TBS solution and staining with BCIP/NBT (5-bromo-4-chloro-3-indol phosphate/nitro blue tetrazolium). Slides were checked every 15-30 minutes. Unstained slides were washed in distilled water and re-treated by the same detection method. After rinsing with distilled water, sections were counterstained with eosin and coverslipped with crystal mount (DAKO). For quality control, positive and negative DNA probes (DAKO kit) were used in every stain.

RESULTS

Histological evaluation ; Fifteen cases of Group A had acanthosis (100%). Thirteen (86.7%) intraepithelial capillary loops, 12(80.0%) koilocytosis, 8(53.3%) dyskeratosis, 6(40.0%) basal cell hyperplasia and 5 (33.3%) multinucleation were observed in Group A. In Group C, all 26 cases showed 100% of acanthosis and koilocytosis. Twenty five(96.2%) intraepithelial capillary loops, 14(53.8%) dyskeratosis, 12(46.2%) basal cell hyperplasia and 9(34.1%) multinucleation were observed in

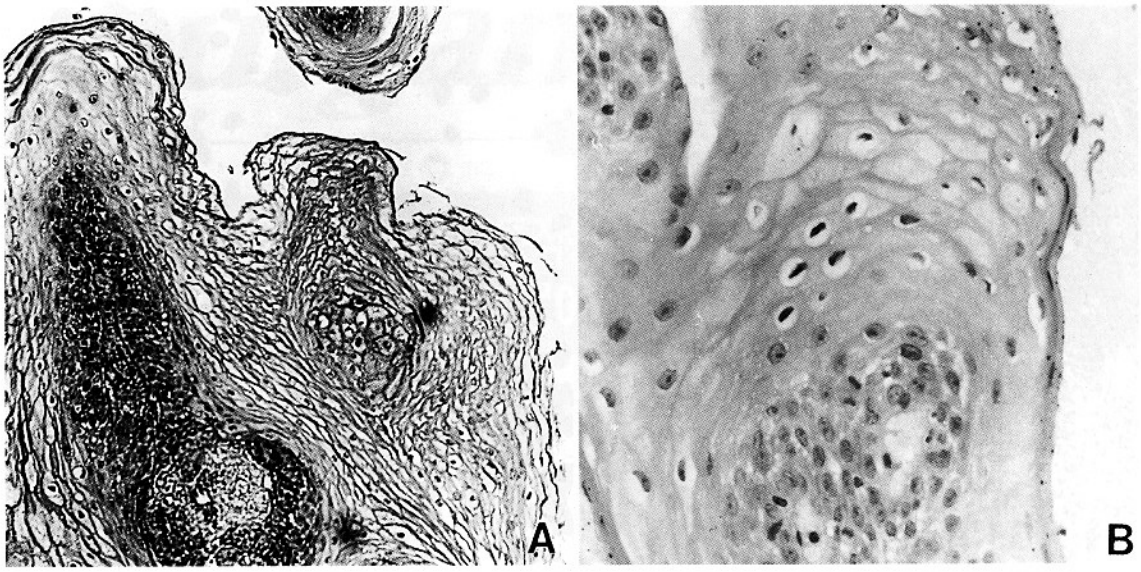

Fig. 1. (A) An exophytic lesion with acanthosis and intraepithelial capillary loops is seen. (H & E)
(B) Koilocytosis is demonstrated. (H & E)

Group C (Fig. 1 and Table 2). Thus, all 41 cases (15 Group A and 26 Group C) had acanthosis (100%). Koilocytosis and intraepithelial capillary loops were also observed in 92.7% in these cases. Dyskeratosis, basal cell hyeprplasia and multinucleation were 53.7%, 44.0% and 34.1% in frequency. Grading by "HPV score" from Toki and Yajima (1987), 12 cases of 15 in Group A and all of Group C scored more than 6 of a total 10 points and they coincided with the diagnosis of HPV infection.

***In situ* hybridization**; Twelve cases (34.3%) of 35 total examined squamous cell lesions of the esophagus showed positive signals for wide spectrum HPV DNA probe. They were 11 cases of 25 SCCs (44.4%); 6 cases of 10 in Group A (60%) and 5 cases of 15 in Group B (33.3%). Only one case of 10 in Group C (10%) showed positive reaction, but it was weaker than Group A or B. Variation of positive intensity was present, so they were divided into trace, 1+ and 2+. Cases which showed vague nuclear positive reactions with equivocal reaction in cytoplasms and on background were interpreted as trace. The trace cases were excluded and only 1+ and 2+ cases were included in the positive group (Table 3, Fig. 2 and 3).

In correlation of HPV-positive cases with histologic differentiation, 9 cases of 11 SCCs (81.8%) which reacted positively with HPV DNA by ISH showed moderate differentiation; six positive reaction cases of Group A were 1 well differentiated and 5 moderately differentiated SCC, and 5 positive reaction cases of Group B

Table 2. Frequency of histologic parameters suggesting HPV infection

Histologic parameters	Group A (n=15) No.(%)	Group C (n=26) No.(%)	Total (n=41) No.(%)
Koilocytosis	12(80.0)	26(100.0)	38(92.7)
Bi- or multinucleation	5(33.3)	9(34.1)	14(34.1)
Dyskeratosis	8(53.3)	14(53.8)	22(53.7)
Intraepith. capillary loops	13(86.7)	25(96.2)	38(92.7)
Basal cell hyperplasia	6(40.0)	12(46.2)	18(44.0)
Acanthosis	15(100.0)	26(100.0)	41(100.0)

Table 3. Expression rate of HPV DNA in squamous cell carcinoma and squamous papilloma of esophagus using *in situ* hybridization for wide-spectrum HPV probe

Group (No. of Cases)	–	Trace	1+	2+	Positivity (%)
A (10)	2	2	3	3	60.0
B (15)	9	1		5	33.3
C (10)	8	1	1		10.0
Total (35)	19	4	4	8	34.3

*A ; SCC with evidences of HPV infection
B ; SCC without evidences of HPV infection
C ; SP

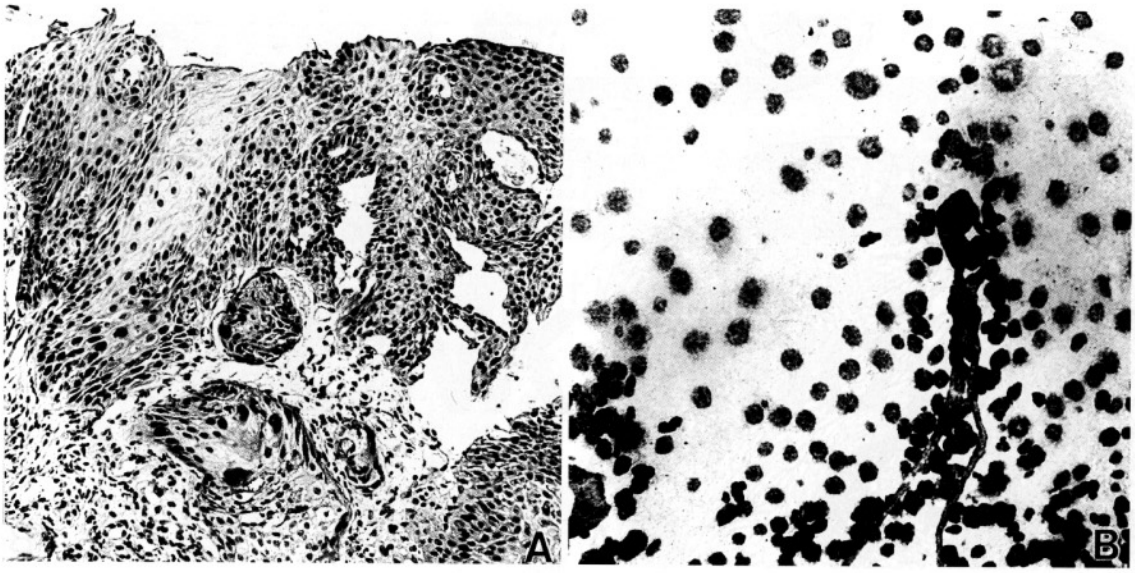

Fig. 2. (A) Malignant squamous cell clusters and adjacent acanthotic squamous mucosa are seen. (H & E)
(B) Positive nuclear signals on *in situ* hybridization study for wide-spectrum HPV probe in squamous cell carcinoma area are noted.

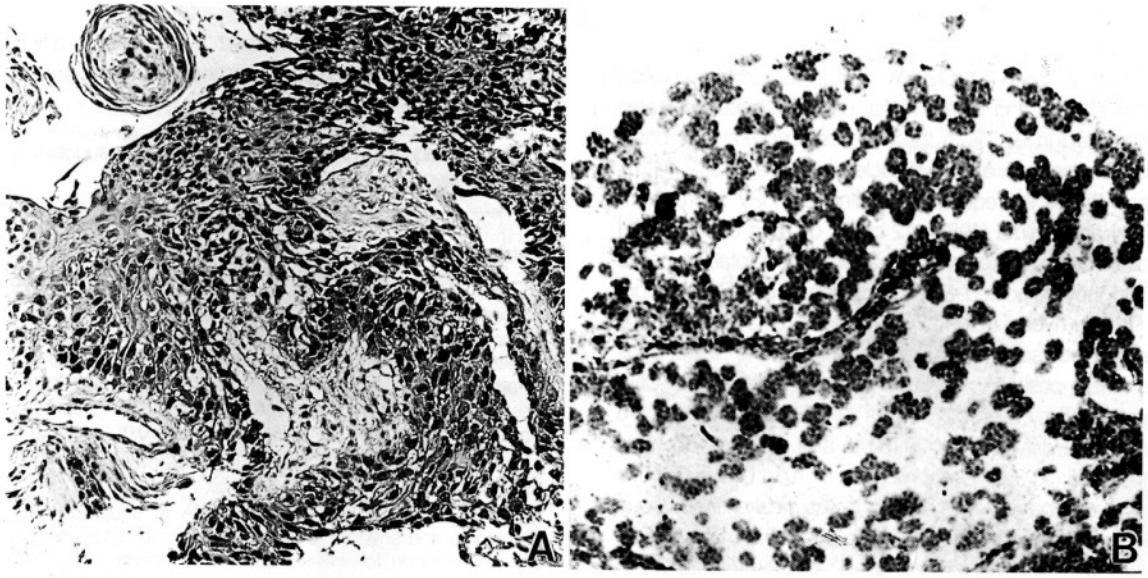

Fig. 3. (A) Squamous cell carcinoma with infiltrative growth is found. (H & E)
(B) Nuclear expressions for wide-spectrum HPV probe on *in situ* hybridization are observed.

were 1 well differentiated and 4 moderately differentiated SCC.

DISCUSSION

Squamous cell papilloma of the esophagus is a rare lesion ; it's prevalence is 0.01-0.04% in Western countries and peak incidence is the fifth decade. It is found more often in men than women. There is considerable controversy regarding the etiology of esophageal squamous papilloma and little is known. Presented hypotheses for pathogenesis are as first, over regener-

ation response to chronic mucosal irritation (Quitadamo and Benson, 1988 ; Odze et al., 1993), second, associated with virus (Politoske, 1992). Furthermore genetic association (Carr et al., 1994) is also reported. The first theory is supported by the common association of SPs with gastro-esophageal reflux, esophagitis and hital hernia, and by the high prevalence of SPs in the lower level of the esophagus (Quitadamo and Benson, 1988). Further evidence in support of this theory is derived from animal studies, in which SPs can be induced by ingestion of specific chemical irritants like N-methylnitrosoamine (Stinson et al., 1978). No viral inclusion bodies were detected by electromicroscopic findings (Toet et al., 1985) and no reactivity of the virus culture in tissue was observed (Colina et al., 1980). However, Politoske (1992) reported that SP showed no recovery after removal of etiology, such as gastro-esophageal reflux, suggesting the possibility of other pathogenesis.

Apparently the true prevalence of HPV infection of the esophageal SP is still underestimated. However, intraepithelial capillary loops, koilocytosis, acanthosis, dyskeratosis, multinucleation and basal cell hyperplasia, which are seen in HPV infection of the uterine cervix have been observed, indicating possible HPV infection. In our study of 26 SPs, these histologic parameters suggestive of HPV infection were very frequently observed (koilocytosis and acanthosis : 100%, intraepithelial capillary loops : 96.2%, dyskeratosis : 53.8%, basal cell hyperplasia : 46.2%, multinucleation : 36.5%). These correspond well to histologic diagnostic criteria that Toki and Yajima (1987) suggested.

Winkler et al. (1985) reported that 31% of SPs having histological findings of HPV infection showed positive reactions to HPV antigens in immunohistochemical staining. These cases had a history of chronic esophageal irritaion. They documented an association between HPV and SP, but also an association with chronic irritation and mucosa injury. Politoske (1992) reported one case of SP using DNA *in situ* hybridization techniques. The strains of HPV identified are types 6 and 11 which are similar to those found in the oropharynx and genital tract, raising the possibility of sexual transmission. He also thought that an isolated single SP lesion located in the lower esophagus appeared more characteristic of chronic gastro-esophageal reflux as an etiology, but multiple papilloma found in higher levels of the esophagus seemed to favor involvement of HPV. Odze et al. (1993) reported 50% positivity of the HPV DNA in his 38 cases of PCR test; the most common type was 16, and he proposed that multifactorial etiology in which the synergistic action of mucosal irritation and HPV for the development of SP is involved. In the report of Carr et al.(1994), using ISH and PCR, only one case of 23 SPs showed positive reaction to HPV type 6 and 11. They suggested that SPs of the human esophagus are not associated with HPV and that other pathogenetic mechanisms, such as mucosa injury and repair are more important in the etiology of these lesions. In our study, only one case of 10 (10%) SPs showed a weak positive reaction. These findings are more consistent with the hypothesis that not only HPV infection but also mutifactorial etiology, such as mucosa irritation, act synergistically in the development of human SP.

Chang et al. (1990b) documented that 54.8% of koilocytosis and more than 20% frequency of acanthosis, dyskeratosis and mutinucleation in SCC in China. In the research for esophageal SCC in Koreans, Kim et al.(1992a) reported that 50.0% of koilocytosis in the adjacent area of SCC and acanthosis, dyskeratosis, multinucleation, hyperkeratosis were 34.6%, 30.8%, 15.4 % and 7.7% in frequency. We evaluated these histologic parameters in Group A (SCC with evidences of HPV infection) and they were very frequently observed (acanthosis : 100.0%, intraepithelial capillary loops : 86.7%, koilocytosis : 80.0%, dyskeratosis : 53.3%, basal cell hyperplasia : 40.0% and multinucleation : 33.3%).

Immunohistochemical staining for HPV antigens in esophageal SCC was reported to be 13.0-69.2% of variable positivity in previous reports (Hille et al., 1983 ; Mori et al., 1989 ; Kim et al., 1992a). Hille et al. (1983) reported that the prevalence rate of the HPV antigen of SCC in South Africa was 33% (mid-risk area for SCC). In the report of Mori et al. (1989), the prevalence rate of HPV antigen was 13% in and adjacent to lesions of SCC in Japan, 19% and 23% in China (high risk area for SCC). In Korea, 69.2% of high detection rate was reported by Kim et al. (1992a).

In the review of the prevalence rate of HPV DNA in esophageal SCC by ISH, Kulski et al. (1986) detected HPV DNA in 5 cases of 10 (50%) esophageal SCCs in Australia by filter ISH using a mixed probe of HPV 11, 13, 16 and 18. Benamouzig et al. (1992) detected HPV DNA in 5 cases of 12 (41.7%) SCCs in France by ISH using HPV 6, 11, 16, 18, 31, 33 probe and the common types found were HPV type 16 and 18. Chang et al. (1990b) examined 51 esophageal SCC in the Luxian district of China (high risk area for esophageal carcinoma) for the presence of HPV 6, 11, 16, 18 by ISH and demonstrated 43.1% positivity of any of those

types. In their report, especially type 16 and 18 were high. In Korea, Kim et al. (1992a) detected HPV 16 and 18 in one case of 10(10%) esophageal SCCs. In our study, demonstration of HPV DNA for wide-spectrum probe was 44.0% in SCC; this result is similar to that of Western countries. Thus, our results suggest that HPV might play a role in the carcinogenesis of the SCC.

HPV is believed to replicate preferentially in keratinized squamous epithelium (Politoske, 1992). Mori et al. (1988) reported that higher HPV expression rates in more differentiated eophageal SCC by using the Avidin-Biotin complex method. In our research, 9 cases of 11 HPV DNA positive reaction SCCs showed moderate differentiation, so, evaluation for the differences of HPV DNA expression rate according to histologic differentiation could not be done.

The malignant potential of SP in the esophagus is controversial. However, Jarett (1987) reported that bovine papilloma virus (BPV 4) caused esophageal SP which transformed to SCC in animal studies. De Borges et al.(1986) has described a patient with an esophageal carcinoma that was apparently preceded by SPs. Van Cutsem et al.(1991) also reported a patient with a large HPV-positive esophageal SP that showed histological evidence of malignant transformation later. Chang et al. (1990a) identified 66.3% of total expression rate by ISH using HPV type 11, 16, 18 DNA probe in precancerous lesion and SCC of the esophagus. In their cases, the detail expression rate were 22.2% in simple hyperplaisa, 50% in mild dysplasia, 80.6% in moderate dysplasia, 67.8% in severe dysplasia, and 66.7% in SCC. These reports raised speculation regarding the potential for HPV infection in the role of dysplastic and malignant transformation of esophageal squamous lesions. In our study, the SCC having histological evidence of HPV infection in adjacent areas (Group A) showed 60% and SCC without evidences of HPV infection (Group B) showed 33.3% of HPV DNA expression rate. Although there was somewhat of a limitation in accessing the accuracy, because all the cases were biopsied materials, these support the evidence implicating HPV in the genesis of esophageal SCC. SP alone (Group C) showed low expression rate (10%) of HPV DNA by ISH, however, we still believe that somehow HPV involves in the pathogenesis of SP, either as promotor in association with other factors or as prime etiology, because cases showed so frequent histologic parameters suggestive of HPV infection. In conclusion, we emphasize the significant role of HPV infection in the development of benign and malignant squamous cell lesions of the esophagus, especially SCC. Further studies with HPV subtype are required to clarify more precisely the association of HPV infection with the development of SP and the cacinogenesis of SCC in the esophagus.

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