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HPV type 16 in conjunctival and junctional papilloma, dysplasia, and squamous cell carcinoma

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Abstract

***Aims*—To clarify the role of human papillomavirus (HPV) infection in the development of papilloma, dysplasia, squamous cell carcinoma, and basal cell epithelioma arising from the eyelids, including the tunica conjunctiva palpebrum (conjunctiva), its junction to epidemis of eyelid skin (junction), and eyelid skin.**

***Methods*—Sixteen cases of papilloma, four of dysplasia, four of squamous cell carcinoma, and 12 of basal cell epithelioma were examined using formalin fixed and paraffin embedded samples. Detection of HPV-DNA was performed by PCR-RFLP and in situ hybridisation (ISH) methods.**

***Results*—HPV-16 was detected in 12/16 papillomas (75%), 2/4 dysplasias (50%), and 1/4 squamous cell carcinomas (25%) but in none of the basal cell epitheliomas. No other HPV subtypes were found. ISH assay showed positive signals in only two cases of dysplasia and squamous cell carcinoma. The mean age of HPV-16 positive dysplasia and squamous cell carcinoma cases (81·7 years) was significantly higher than that of HPV-16 positive papilloma cases ($p<0·01$).**

***Conclusions*—Based on the presence of HPV-16 in both benign and malignant lesions and the age distribution, it seems likely that HPV-16 alone may be incapable of causing development of conjunctival and junctional dysplasia and squamous cell carcinoma, and that any correlation between the papilloma-squamous cell carcinoma sequence and HPV infection may be due to rare events.**

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Keywords: HPV, conjunctiva, papilloma, squamous cell carcinoma.

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The human papillomavirus (HPV), a representative member of the oncogenic viruses, is composed of closed circular double stranded DNA of approximately 8 kb in length.[1] Its occurrence is closely related to squamoproliferative lesions of the cervix, anogenital region, skin, and upper respiratory and digestive tracts.[2-4] Over 50 types of HPV have been identified to date[5] and some have been shown to play an important role in the development of tumour lesions. For example, in muco-squamous epithelium of uterine cervix and vulva, HPV-6 and HPV-11 induce benign papillomatous lesions, and HPV-16 and HPV-18 are closely linked with progression to malignancy.[6 7]

Earlier studies have implicated HPV infection in the pathogenesis of conjunctival papillomas because of the frequent finding of koilocytotic features in these lesions. More recently, the presence of HPV capsid antigens and DNA sequence has been documented in conjunctival neoplasms, including papillomas, dysplasia, and squamous cell carcinomas.[8 9] However, no detailed examination of the correlation between HPV infection and benign and malignant lesions originating from the eyelid area has so far been described.

In the present study, to clarify the exact role of HPV infection in tumour development, papillomas, basal cell epitheliomas, dysplasia, and squamous cell carcinomas arising from eyelids—including the tunica conjunctiva palpebrum, its junction between the conjunctival mucosa and the epidermis of eyelid skin, and the eyelid skin itself—were examined using molecular biological and clinicopathological methods.

Methods

CASE SELECTION AND DNA EXTRACTION

Sixteen cases of papilloma, 12 of basal cell epithelioma, four of dysplasia, and four of squamous cell carcinomas surgically removed from the eyelid area, including the tunica conjunctiva palpebrum (conjunctiva), its junction to eyelids epidermis (junction), and the eyelid skin, were selected from patient files of the Kitasato university hospital for the years 1979 to 1994. All resected samples had been fixed in 10% formalin and embedded in paraffin.

DNA samples for the detection of HPV-DNA were obtained by scraping off tumour cells identified on several serial 10 μm thick paraffin sections. DNA extraction was performed through phenol/chloroform treatment as described previously.[10]

POLYMERASE CHAIN REACTION ASSAY

For the detection of HPV-DNA, consensus primers for the HPV L1 region, L1C1 and L1C2, published by Yoshikawa *et al*,[11] were used. These can differentiate nine species of HPV, including types 6, 11, 16, 18, 31, 33, 42, 52, and 58. Polymerase chain reaction (PCR) mixture (10 μl) containing 1 ng template DNA, 100 nM of each primer, and 0·5 unit of Taq DNA polymerase (Takara) was prepared. The PCR procedure consisted of 45 cycles of 30

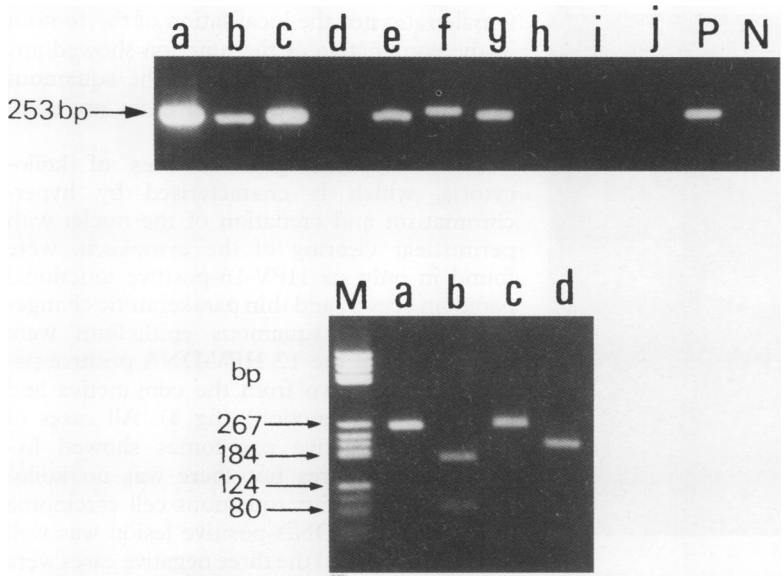

Figure 1 Amplification of HPV DNA by consensus primers for the L1 region (upper panel) and HPV typing by restriction fragment length polymorphism analysis (lower panel). Upper panel: lane A, squamous cell carcinoma case; lanes b–d, dysplasia cases; lanes e–i, papilloma cases; lane j, basal cell epithelioma case. P=positive control; N=negative control. Lower panel: lane a, uncut; lane b, Dde I digestion; lane c, Rsa I digestion; lane d, Hae III digestion. M=molecular marker (DNA molecular weight marker V, Boehringer Mannheim).

seconds at 94°C, two minutes at 48°C and at 72°C associated with a predenature step of two minutes at 94°C, and postextension for 10 minutes at 72°C.

The PCR products were digested with *Rsa* I, *Dde* I, and *Hae* III, and HPV subtypes were determined by restriction fragment length polymorphism (RFLP).

The quality of the DNA extracted was confirmed before starting the study using β globin gene specific primers.[12] PCR was performed as a replicate or triplicate assay. DNA available from uterine cervical carcinoma cases and proven to be positive for HPV-16 or HPV-18 was used as positive control, and water instead of DNA was used for negative control.

IN SITU HYBRIDISATION PROCEDURE

The probe used for the in situ hybridisation was a fluorescein labelled DNA probe for wide spectrum HPV DNA (Dako), and an in situ hybridisation detection system was applied. Briefly, samples were digested with 0·8% pepsin/0·2 N HCl, and hybridisation was carried out at 37°C overnight. Hybridisation signals were detected according to the manufacturer's instructions. As positive control, fluorescein labelled human genomic DNA was used, and a pUC plasmid vector was examined as negative control.

STATISTICS

Statistical analysis of data was performed using the Mann-Whitney U test.

Results

PCR ASSAY

Amplified specific HPV DNA was detected in 12 of the 16 papilloma cases (75%). Five lesions of these 12 HPV-DNA-positive cases were located in the conjunctiva, and seven were from the junction. Two of four dysplasia cases (50%) (one from the conjunctiva, and the other from the junction), and one of four squamous cell carcinoma cases (originating from the junction) also showed the presence of HPV-DNA (fig 1). No HPV-DNA was noted in basal cell epithelioma cases.

RFLP assay revealed that all amplified HPV DNAs were from HPV-16, since all PCR products digested with *Dde* I were cleaved into 169 base pairs (bp) and 84 bp fragments and *Hae* III digestion produced 200 bp and 53 bp fragments, whereas a restriction site for *Rsa* I was absent (fig 1). None of the other HPV subtypes was detected in this study.

IN SITU HYBRIDISATION ASSAY

In situ hybridisation examination was performed for all HPV-DNA positive cases determined by PCR assay to clarify the location of HPV in tumour tissues. Only two cases (one a dysplasia case and the other a squamous cell carcinoma case) showed positive hybridisation signals in the nuclei of the tumour cells (fig 2). However, no hybridisation signal could be detected in any of the papilloma cases or in the remaining dysplasia case.

CLINICOPATHOLOGICAL ANALYSIS

As shown in table 1, the age of the papilloma cases ranged from 14 to 73 years (mean 38·1).

Table 1 Data summary for conjunctival and junctional papilloma cases

Case No	*Age and sex*	*Lesion*	*HPV type**	*ISH*	*Koilocytosis*	*Parakeratosis*	*Hyperkeratosis*
1	23, F	Conj	16	–	–	–	–
2	22, F	Conj	16	–	–	+	–
3	24, M	Conj	16	–	–	+	–
4	53, M	Conj	16	–	–	–	–
5	53, F	Conj	16	–	–	–	–
6	23, F	Jun	16	–	+	+	–
7	14, M	Jun	16	–	+	+	–
8	25, F	Jun	16	–	+	–	–
9	47, F	Jun	16	–	+	–	–
10	41, F	Jun	16	–	+	–	–
11	40, F	Jun	16	–	–	+	–
12	40, M	Jun	16	–	+	+	–
13	59, M	Jun	–	ND	–	–	+
14	73, F	Jun	–	ND	–	–	+
15	51, F	Jun	–	ND	–	–	+
16	22, F	Jun	–	ND	–	–	+

* HPV subtype was determined by PCR-RFLP assay. ISH=in situ hybridisation; Conj=conjunctiva; Jun=junction between conjunctival epithelium and eyelid epidermis; ND=not done.

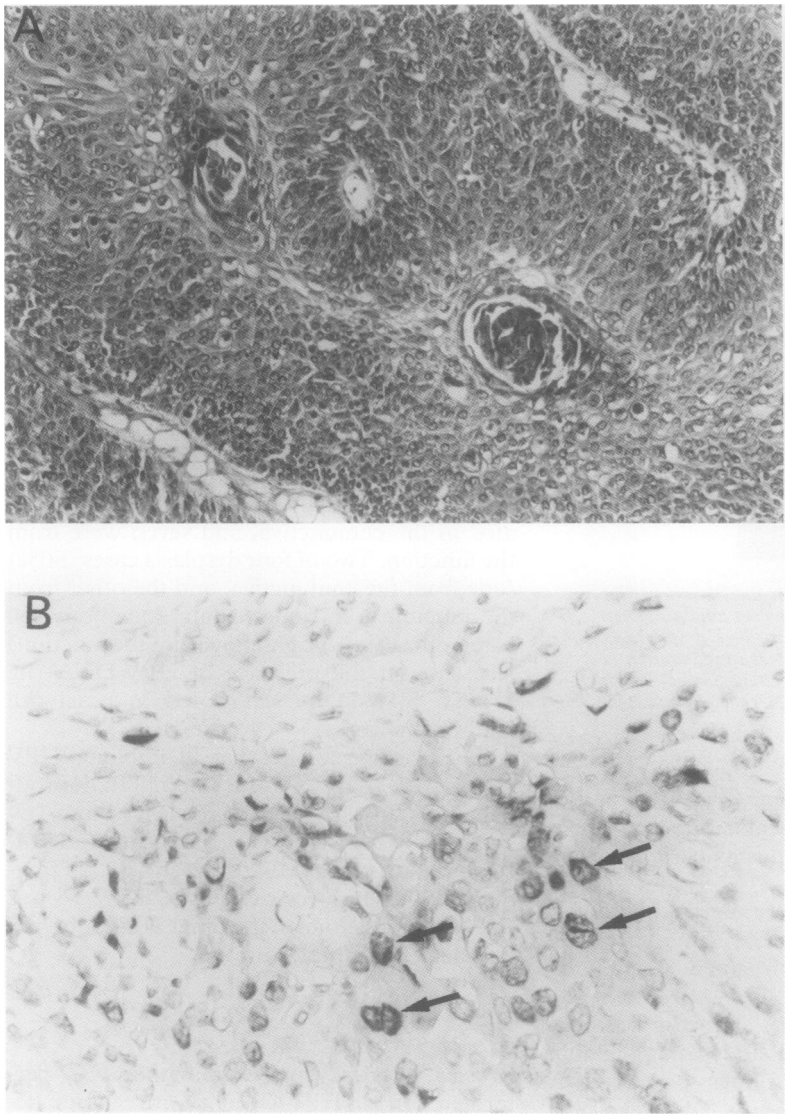

Figure 2 (A) HPV-16-positive squamous cell carcinoma (case 3). Note the apparent cancer pearls and papillomatous growth. H and E, × 175. (B) In situ hybridisation on a section of squamous cell carcinoma (case 3). Positive hybridisation signals are evident in the nuclei of tumour cells (arrows). × 350.

Table 2 Data summary of SCC and dysplasia cases

Case no	*Age and sex*	*Lesion*	*Histology*	*HPV type**	*ISH*
1	82, F	Jun	Mod SCC	–	ND
2	75, F	Jun	Mod SCC	–	ND
3	85, F	Jun	Well SCC	16	+
4	70, M	Jun	Poor SCC	–	ND
5	83, F	Jun	Dysplasia	16	–
6	77, M	Conj	Dysplasia	–	ND
7	49, M	Conj	Dysplasia	–	ND
8	77, M	Conj	Dysplasia	16	+

* HPV subtype was determined by PCR-RFLP assay. ISH = in situ hybridisation; Conj = conjuctiva; Jun = junction between conjunctival epithelium and eyelid epidermis; Well = well differentiated; Mod = moderately differentiated; Poor = poorly differentiated; SSC = squamous cell carcinoma; ND = not done.

For the HPV-16-positive cases the age range was from 14 to 53 years (mean 33·8) and for negative cases from 22 to 73 years (mean 51·3), with no significant difference between the ages ($p = 0·16$). In contrast, the ages of dysplasia and squamous cell carcinoma cases ranged from 49 to 85 years (mean 74·8). The mean age of HPV-16-positive dysplasia and squamous cell carcinoma cases (81·7 years) was significantly higher than that of the HPV-16-positive papilloma cases ($p<0·01$). Neither the male to female ratio nor the localisation of the tumour in the conjunctiva or the junction showed any link to HPV-16. The data on the squamous cell carcinoma and dysplasia cases are summarised in table 2.

The histopathological features of koilocytosis, which is characterised by hyperchromatism and crenation of the nuclei with perinuclear clearing of the cytoplasm, were found in only six HPV-16-positive junctional papilloma cases, and thin parakeratotic changes in proliferating squamous epithelium were noted in six of the 12 HPV-DNA-positive papilloma cases (two from the conjunctiva and four from the junction) (fig 3). All cases of HPV-DNA-negative papillomas showed hyperkeratotic figures but there was no koilocytosis. Of the four squamous cell carcinoma cases, the HPV-DNA-positive lesion was well differentiated, and the three negative cases were moderately or poorly differentiated.

Discussion

The eyelid is anatomically composed of the tunica conjunctiva palpebrum, its junction to the eyelid epidermis, and the eyelid skin itself. It is known that conjunctival papillomas frequently contain goblet cells—usually showing a tendency to epidermidalisation and keratinisation of moderate degree—and that papillomatous eyelid skin consists of hyperplastic squamous epithelium, which shows acanthosis, parakeratosis, and hyperkeratosis. In the present study, HPV-16-positive junctional papilloma cases showed koilocytotic and thin parakeratotic features, while HPV-16-negative cases showed hyperkeratotic figures without koilocytosis. Considering that HPV-16 is capable of infecting the conjunctival mucosa, it may thus be possible to determine histopathologically the origin (mucosa or epidermis) of junctional papillomas and the presence or absence of HPV.

Earlier studies have demonstrated a close linkage between conjunctival papillomas and HPV-6 and HPV-11 infection.[13,14] McDonnell *et al*,[15] using in situ hybridisation, found that 15 of 23 cases of conjunctival papilloma (65·2%) had HPV-6. Moreover, 13 out of 15 positive cases (86·7%) were under the age of 30 years, suggesting that infection with HPV-6 may be responsible for most of the conjunctival papillomas occurring in children and young adults. In the present PCR-RFLP assay study, HPV-16 was detected in conjunctival and junctional papillomas, but not HPV6/11. The PCR assay is able to detect even a single copy of target DNA, although there are serious problems with possible contamination. To avoid false positive or negative results, we conducted PCR as a replicate or triplicate assay, also providing several negative controls. The RFLP assay applied in this study is very reliable for determination of HPV subtyping, since the various restriction enzyme sites in amplified specific HPV DNA L1 region sequences clearly differ with the HPV subtype. Therefore, it seems possible that HPV-16 is causally related to conjunctival papillomas. The discrepancy between this and

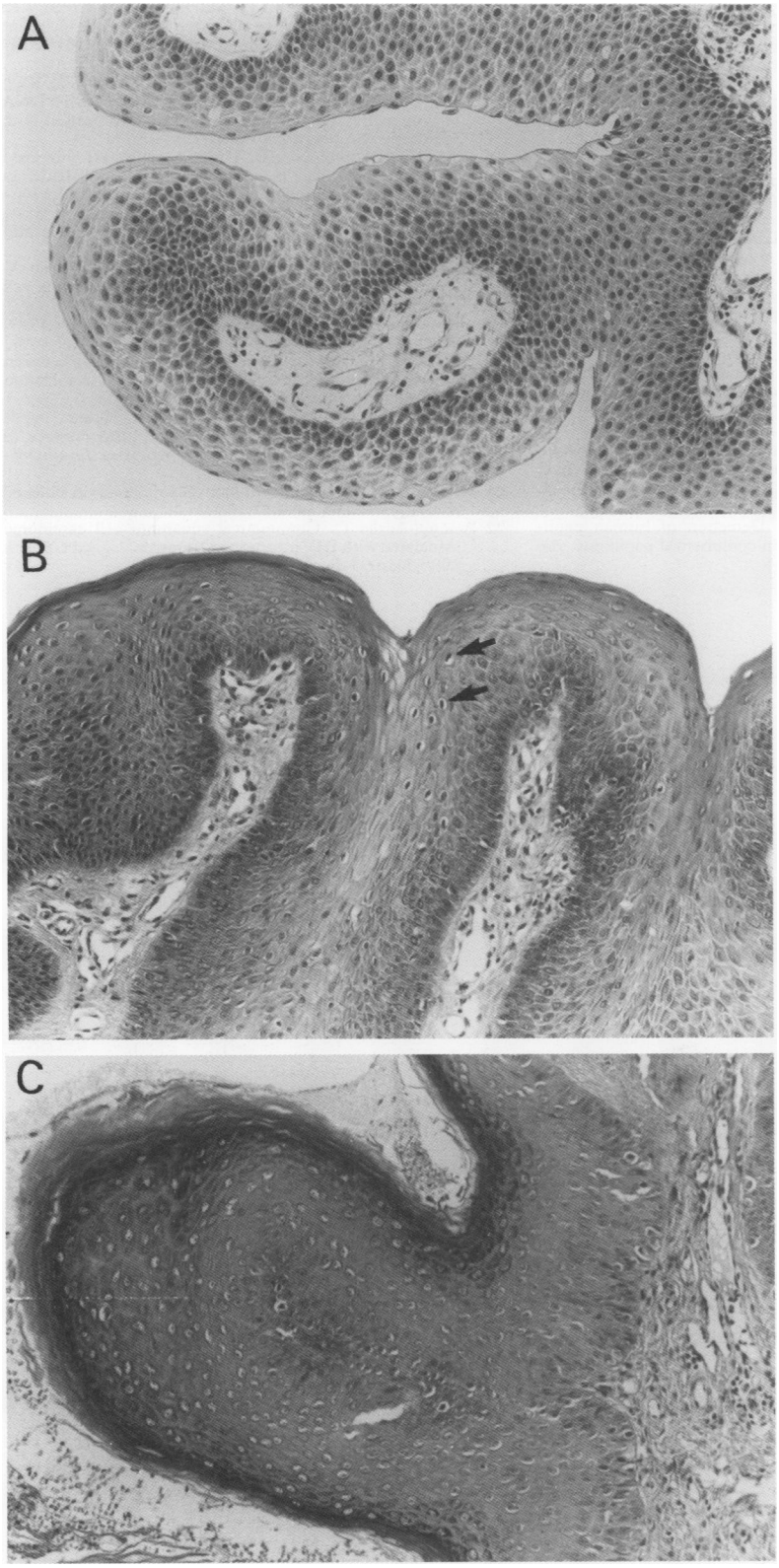

Figure 3(A) HPV-16-positive conjunctival papilloma (case 5) showing typical histology, including the presence of goblet cells and a tendency to epidermidalisation. H and E, × 175. (B) HPV-16-positive junctional papilloma (case 6). Koilocytotic (arrows) and thin parakeratotic (upper left side) features are present. H and E, × 175. (C) HPV-negative junctional papilloma (case 16) showing papillomatosis, hyperkeratosis, and the absence of koilocytosis. H and E, × 175.

previous studies may be due to the age of cases investigated, since half of the HPV-16-positive papilloma cases in this study were older than 40 years.

In order to detect HPV signals by in situ hybridisation using radioactive DNA probes, it has been estimated that about 50 to 100 viral copies per cell are required.[16] False negative reactions could therefore be caused by low levels of viral replication, as well as inappropriate tissue fixation and processing. This would explain the discrepant results between polymerase chain reaction and in situ hybridisation assays in this study, since the PCR-RFLP technique is more sensitive.

It has been suggested that HPV alone cannot completely transform primary human keratinocytes or other cells,[17] but that addition of mutationally activated *v*-Ha-*ras* to HPV-16 immortalised human cervical cells results in full malignant transformation.[18] zur Hausen[19] has suggested that HPV may act as a promoter-like agent in synergism with carcinogenic initiators such as cigarette smoke or herpes simplex virus in the development of cervical neoplasia.

The oncogenic role of HPV in conjunctival neoplasms has been recently discussed. McDonnell et al,[20] using polymerase chain reaction and dot blot hybridisation methods, showed that HPV16 was present in 37 out of 42 conjunctival epithelial lesions (88·1%), including mild to severe dysplasias and invasive carcinomas, suggesting that the interaction of HPV with ultraviolet light or some other element plays an important role in the development of neoplasia at this site. In addition, Wilson and Ostler[21] stated that conjunctival papilloma can be divided into two groups, infected and non-infected, the non-infected papillomas—probably related to ultraviolet light exposure—being capable of undergoing malignant transformation. Recently, Ateenyi-Agaba[22] indicated that the combination of human immunodeficiency virus (HIV) induced immunosuppression, HPV infection, and intense exposure to ultraviolet light may accelerate the development of squamous cell carcinoma. Our results show that HPV-16 infection is associated with dysplasia and squamous cell carcinoma but not basal cell epithelioma. In addition, most of the dysplasia and squamous cell carcinoma cases were older than 70 years, with a mean age greater than that of HPV-16-positive papilloma cases. Based on the presence of HPV-16 in both benign and malignant lesions and its age distribution, it seems likely that HPV-16 alone may be incapable of inducing conjunctival and junctional dysplasia and squamous cell carcinoma, and that any correlation between the papilloma-squamous cell carcinoma transition and HPV infection may be due to rare events. Further studies are indicated to explore the relation between HPV-16 infection and other carcinogenic agents in the development of conjunctival and junctional dysplasia and squamous cell carcinoma.

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