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LABORATORY STUDY

Detection of human papillomavirus in pterygium and conjunctival papilloma by hybrid capture II and PCR assays

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Abstract

Aim **To elucidate the putative role of human papillomavirus (HPV) infection in pterygium and conjunctival papilloma.**

Methods **Hybrid capture II (HC-II) and polymerase chain reaction (PCR) assays were performed to detect HPV in pterygium (42 samples obtained from 40 patients) and conjunctival papilloma (8 samples from 6 patients). The amount of HPV DNA was evaluated by measurement of relative light units (RLUs) on a luminometer.**

Results **All papilloma samples were positive for HPV DNA by PCR and HC-II. The RLU values for specimens of recurrent and re-recurrent papilloma were markedly higher than those for specimens of primary lesions. HPV was detected by PCR in 2 of 42 (4.8%) *β*-globin-positive pterygium specimens, whereas HC-II showed that HPV was negative in all pterygium samples.**

Conclusions **Our results support the hypothesis that HPV DNA is associated with the pathogenesis of conjunctival papilloma, but not pterygium. RLU measurement by HC-II may serve as a marker for evaluating the activity of HPV in conjunctival tumours.**

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Keywords: human papillomavirus; pterygium; papilloma; PCR; hybrid capture assay; HC-II

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Introduction

Pterygium and papilloma are benign conjunctival tumours that can be treated by surgical excision. However, recurrences are frequently observed, and recurrent lesions tend to grow more rapidly than primary lesions.[1] Thus, it is important to clarify the mechanisms of development of these lesions. Although the aetiology of pterygium and papilloma has not been established, human papillomavirus (HPV) is thought to be one of the causative factors.[2–5]

HPV is a family of double-stranded circular DNA viruses, of which more than 80 types have been identified.[6] HPVs have oncogenic potential and contribute to the progression of neoplastic conditions. HPV DNA has been detected in papillomas, dysplasia, and cancers in the cornea and conjunctiva.[7] The association of HPV with the pathogenesis of conjunctival papilloma is well established, as many investigators have demonstrated the presence of HPV DNA in conjunctival papilloma using polymerase chain reaction (PCR).[8,9] Also, several studies have shown that HPV DNA can be detected in pterygium by PCR.[3,10,11] However, the proportion of cases that are positive for HPV DNA shows marked variation; HPV DNA has been detected in up to 50% cases of pterygium, whereas other studies have failed to detect any.[12] Therefore, the involvement of HPV in the pathogenesis of pterygium remains controversial.

To address this issue, we attempted to measure the amount of HPV DNA in conjunctival papilloma and pterygium. The second-generation hybrid capture (HC-II) assay is a non-radioactive, reproducible, relatively rapid liquid hybridization assay, with a sensitivity similar to that of PCR.[13,14] It is based on the measurement of relative light units (RLUs) as a semiquantitative index of the amount of HPV DNA.[15] Some previous studies

have shown that the RLU level is correlated positively with disease severity.[16] As studies of conjunctival papilloma using PCR have revealed a high rate of HPV detection, it is probable that this lesion is suitable for investigation by HC-II to clarify the magnitude of the RLU value, which would provide further information for clarifying the role of HPV in its pathogenesis. In the present study, we applied the HC-II assay for detection of HPV DNA in conjunctival pterygium and papilloma, and evaluated the possible correlation between the amount of HPV DNA and the clinical profile of these conjunctival tumours.

Materials and methods

After obtaining the approval of the institutional review board, 42 specimens of pterygium were obtained from 42 patients: 22 male and 18 female, age range 39–82 years, mean age 64 years. Eight specimens of conjunctival papilloma were obtained from six patients—five patients at the first occurrence (primary specimen), and one patient (case 6) at the time of primary, at recurrence 2 months after the first excision (recurrent specimen), and at re-recurrence 4 months after the first excision. For PCR and HC-II analyses, 10 mg each of tissue specimens were used. The rest of sample was used for histological review on haematoxylin- and eosin-stained sections for confirmation of the diagnoses. Research procedures were in accordance with the Declaration of Helsinki Principles. All patients were informed of the aim of this study and gave their consent for use of their tissue samples.

Polymerase chain reaction

Formalin-fixed paraffin blocks were incubated with 200 μg/ml proteinase K in 10 mM Tris (pH 7.0)–1 mM EDTA at 65°C for 3 h, spun, and the supernatant was used for PCR assay. Primers for a fragment of the β-globin gene served as an initial control to assess the quality and quantity of the template DNA. To detect the presence of HPV DNA, PCR was performed with the generally accepted consensus GP5+/GP6+ primers.[17] The samples were heated at 95°C for 4 min, followed by 40 cycles of amplification: denaturation at 94°C for 1 min, annealing at 40°C for 2 min, and extension at 72°C for 1.5 min. A 15 μl volume of the PCR product was run in a 2% agarose gel, and stained with ethidium bromide.

Hybrid capture assay II

Surgically obtained samples were immediately and directly subjected to HC-II system (Digene, Gaithersburg, MD, USA) and assayed in accordance with the manufacture's instruction manual. This assay is based on sandwich hybridization followed by a non-radioactive alkaline phosphatase reaction with chemoluminescence using microplates supplied the HC-II kit. RLUs were calculated as follows: LU of sample/LU of positive control. The standard cut-off point (1 RLU) was considered positive for the presence of HPV DNA. HPV-11 and -16 DNA (1 pg/ml each) were used as a positive control for both low- and high-risk probes, respectively.

Results

The β-globin fragment was amplified by PCR in all of the 47 specimens (42 cases of pterygium and 7 cases of

Table 1 Details of specimens used

Diagnosis	*Patient number*	*Specimen type*	*Age (years)*	*Sex*	*HPV detection*		*RLUs measured by HC-II*	
					PCR	*HC-II*	*Low-risk*	*High-risk*
Papilloma	1	Primary	76	M	(+)	(+)	1.1	0.18
	2	Primary	28	M	(+)	(+)	151.3	0.94
	3	Primary	42	F	(+)	(+)	8.3	0.35
	4	Primary	39	M	(+)	(+)	9.8	0.44
	5	Primary	41	F	(+)	(+)	3.4	0.23
	6	Primary	38	M	(+)	(+)	2.1	0.14
	6	Recurrence			(+)	(+)	50.4	0.21
	6	Re-recurrence			(+)	(+)	242.3	0.98
Pterygium	1	Primary	73	F	(+)	(−)	0.13	0.11
	2	Primary	42	M	(+)	(−)	0.19	0.14
	3–40	Primary			(−)	(−)	<1.0	<1.0
	41	Recurrence	49	F	(−)	(−)	0.09	0.10
	42	Recurrence	71	M	(−)	(−)	0.14	0.16

HC-II = hybrid capture assay II; PCR = polymerase chain reaction; RLUs = relative light units.

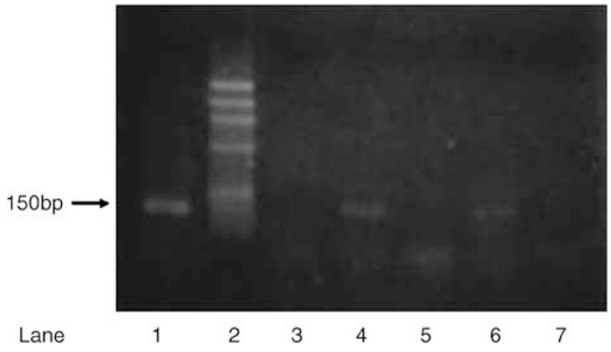

Figure 1 Electrophoresis of PCR products on 2% agarose gel (150 bp sequence of the HPV L1 gene). Lanes 1 and 4 are specimens positive for papilloma (case 1, recurrent and primary lesions, respectively); lane 2, DNA marker; lane 6, HPV-positive specimen of pterygium; lanes 3, 5, and 7, HPV-negative specimens of pterygium.

papilloma). The profiles of the patients and the incidences of HPV in the specimens examined by PCR and HC-II are shown in Table 1. HPV DNA (150 bp) was detected in 2 of the 42 (4.8%) pterygium specimens and in all conjunctival papilloma samples (Figure 1). HC-II assay showed that the primary papilloma specimen was positive for low-risk-type HPV; the RLU values for cases 1, 2, 3, 4, 5, and 6 were 1.1, 151.3, 8.3, 9.8, 3.4, and 2.1, respectively. In case 2 showing high RLU values, the tumour developed rapidly during 1 month, whereas the primary lesion in other cases showed no progression at least for 1 year preoperatively. The recurrent and re-recurrent papilloma specimens taken from case 6 were strongly positive (RLU values 50.4 and 242.3, respectively). All papilloma samples including those from primary and recurrent lesions were negative for high-risk HPV (<1.0). HC-II gave negative results (<1.0) for low- or high-risk-type HPV DNA in all the pterygium specimens.

Discussion

Previous studies have shown good agreement between the results of HC-II and PCR for detection of HPV in premalignant and malignant cervical lesions, and therefore both techniques are considered to be useful for the screening of HPV DNA in these lesions. [18] Similarly, we successfully detected HPV DNA using PCR and HC-II assays in all specimens of conjunctival papilloma. Recently, Sjö *et al*[9] reported that PCR gave positive results for HPV in 86 (81%) of 106 papilloma specimens studied. In addition, HPV was detected in all five papilloma cases studied by Saegusa *et al*,[19] and in 9 of 10 papilloma cases (90%) studied by Gallagher *et al*.[3] The presence of HPV in papilloma has also been confirmed by Southern blotting and *in situ* hybridization.[20] Based on these reports and our data, it is likely that HPV is closely involved in the pathogenesis of conjunctival papillomas.

One of the advantages of HC-II is its ability to estimate viral genome copy number from the magnitude of the RLU value obtained.[14,15] As the RLU value reflects the activity of HPV, it may be closely associated with the progression of papilloma.[15] In fact, our HC-II data showed that the RLU values in recurrent and re-recurrent papilloma specimens were 25 times and 115 times higher, respectively, than in the primary specimen. Moreover, one primary case with severe progression showed extremely high RLU values. Based on our results, we propose that HC-II may be useful for the detection of HPV and evaluation of its activity in conjunctival disease.

In contrast, HPV DNA was not detected in any of the pterygium samples by HC-II assay. The results of PCR assay also showed that 95.2% of pterygium cases were HPV-negative. The low copy number of the HPV genome suggests that the amount of HPV DNA in pterygium is very low. Two recurrent cases of pterygium were negative for HPV DNA by both HC-II and PCR assays. Also, two pterygium cases that were HPV-positive by PCR showed a normal growth pattern with no aggressive features. On the other hand, the RLU values for papillomas showing aggressive growth (case 2) or recurrence (case 6) were markedly high. These results suggest that the amount of HPV reflects the clinical profile of papilloma, whereas this is not the case for pterygium.

Previous studies have shown a wide variation in the detection rate of HPV DNA in pterygium. A recent study has suggested that one possible explanation might be the geographical differences in the frequency of HPV involvement in pterygium.[4] However, in an investigation of a large number of pterygium cases, Sjö *et al*[9] concluded that the low presence of HPV (4/90; 4.4%) detected by PCR does not support the hypothesis that HPV is involved in the development of pterygium. Our data agree with this finding, and suggest that the level of HPV DNA in pterygium is low, implying that the presence of HPV is insufficient for the development of this tumour. As PCR analysis is very sensitive, it detects HPV even when present in tiny amounts, and, in fact, Sjö *et al*[11] have also shown that all pterygium cases that were HPV-positive by PCR were negative by *in situ* hybridization. The development of pterygium may be initiated synergistically by other causative factors, such as chronic exposure to ultraviolet radiation, concomitant infection with herpes virus, and disruption of apoptosis.[5,10,12]

In conclusion, our data suggest that there is a strong association between HPV and conjunctival papilloma, but not pterygium. As the RLU value was linked to the

clinical profile of conjunctival papilloma, HC-II may be a useful tool for monitoring the activity of HPV in conjunctival tumours.

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