

# Detection of Human Papillomavirus in Squamous Cell Carcinoma of Conjunctiva by Nested PCR: A Case Control Study in Iran

Fahimeh Asadi-Amoli<sup>1</sup>, Amir Behzad Heidari<sup>1</sup>, Issa Jahanzad<sup>1</sup>, and Mahmoud Jabbarvand<sup>2</sup>

<sup>1</sup> Department of Pathology, Tehran University of Medical Sciences, Tehran, Iran

<sup>2</sup> Department of Ophthalmology, Tehran University of Medical Sciences, Tehran, Iran

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**Abstract-** Squamous-cell carcinoma (SCC) of the eye conjunctiva is a rare tumor. Its link with immune impairment suggests that infectious agents such as human papillomavirus (HPV) may be involved in the etiology of SCC. We conducted a case-control study on 50 SCC cases (mean age: 65.2) and 50 age frequency-matched control patients with lesion-free, normal conjunctival biopsies (mean age: 63.8) obtained from the cancer registry archive at Pathology Department of Farabi Eye Hospital, Tehran University of Medical Sciences, Tehran, Iran, where SCC has become the most common conjunctival malignancy. MY/GP nested PCR was performed for HPV detection and E6/E7 consensus primers in combination of type specific primers were used in another nested PCR series for HPV typing. HPV DNA was detected in 46 of 50 samples of squamous cell carcinoma and none of the normal biopsies by nested PCR using primer sets of the HPV consensus L1 region (MY/GP). Subsequently, specimens from the 46 positive cases were subjected to specific PCR. Although 630bp amplicon was produced in 44 of 46 samples (E6/E7 primers), none of the specific HPV PCR reactions for HPV DNA type 16, 18, 31 or 33 resulted in the detection of HPV DNA in the 44 SCC specimens of the conjunctiva. Current results confirm the role of HPV in the etiology of conjunctival SCC. The absence of HPV 16, 18, 31 and 33 in conjunctival SCC in this study raise doubts about the role of genital types of HPV in conjunctival carcinomas.

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**Keywords:** Human papillomavirus; Polymerase chain reaction; Squamous cell carcinoma; Conjunctiva

## Introduction

Squamous cell carcinoma (SCC) of the eye conjunctiva is a rare tumor associated with heavy sun exposure, which has greatly increased in some Sub-Saharan African countries, coincident with the spread of HIV (1, 2). An increased risk of SCC has been reported in HIV-positive individuals in the United States (3). The link with immune impairment suggests that infectious agents such as human papillomavirus (HPV) may be involved in the etiology of SCC. HPVs comprise mucosal and cutaneous types, the latter being found in skin carcinomas from patients with Epidermodysplasia verruciformis (EV) (4). HPVs constitute a group of more than 100 different genotypes associated with benign and malignant neoplasms of skin and mucous membranes (5,6). Approximately 40 different HPV genotypes have been detected in the anogenital mucosa (6). On the basis of their epidemiological association with the

development of cervical carcinoma, a group of so called high risk HPV genotypes has been defined. These include HPV genotype 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 (6-9). Other genotypes, such as HPV6, 11, 42, 43, and 44, are classified as low-risk types (10). Low-risk HPV types 6 and 11 have been detected in benign papillomas of the conjunctiva, while studies of SCC have produced inconsistent results. Some studies found HPV types 16 and 18 DNA in SCC tissue biopsies, while others did not (1,11-14).

HPV produces characteristic cytopathic effects in squamous cells recognizable in tissue sections and smears. Definitive identification of the virus is, however, dependent of specific techniques. There are currently four such methods, listed in order of increasing sensitivity: identification of the virus by electron microscopy; staining of the capsule antigen with immunohistochemistry techniques, identification of viral DNA by in situ hybridization; and most sensitive of all,

the polymerase chain reaction (PCR) technique, based on DNA amplification (15).

The use of MY09-MY11 and either GP5-GP6 or GP5+-GP6+ primers in a nested PCR assay has been shown to increase the overall sensitivity compared to that of each primer pair alone (16,17).

Sotlar *et al.* reported a novel PCR assay with the viral E6/E7 oncogenes as the primer target region. In that assay, consensus primers for first-round amplification of a broad spectrum of mucosal HPV genotypes, including high risk HPV genotypes, were combined with type-specific primers for nested PCR amplifications (18). This strategy allows HPV genotyping based on PCR product size.

### Materials and Methods

Between March 2000 and March 2006, we conducted a case-control study on 50 SCC cases (mean age: 65.2) and 50 age frequency-matched control patients with lesion-free, normal conjunctival biopsies (mean age: 63.8) in Pathology Department of Farabi Eye Hospital, Tehran University of Medical Sciences, Tehran, Iran, where SCC has become the most common conjunctival malignancy (19). Formalin-fixed, paraffin-embedded blocks of tissue from SCC cases and control patients were collected, histologically confirmed, and shipped to the PCR Research Laboratory, Pathology Department, Tehran University of Medical Sciences for HPV testing. To minimize the risk of contamination, the collection of the specimens was performed at different days using disposable material. DNA was extracted from each sample in a PCR research laboratory that is exclusively used for this purpose.

#### Isolation of DNA from paraffin-embedded block

8-15 consecutive 15-micron sections from a formalin fixed paraffin embedded block were sliced using disposable blades and gloves to avoid sample contamination, and placed into Eppendorf tubes. 1 ml xylene (Merck, Darmstadt, Germany) was added, vortex mixed, incubated on a shaker heater at 55°C, 120 rpm for 15 min. Then the sample was spun down for 10 min in Eppendorf ultramicrofuge at 14000 rpm. The supernatant was pipet off and discarded. This step was repeated again. Then the same step was done twice with 1 ml of 100% ethanol (Merck, Darmstadt, Germany). After the last step ethanol was removed and the pallet was allowed to air dry. Extraction of total DNA was performed using the QIAmp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's

instructions. In brief, forty microliters of QIAGEN protease stock solution and 400 µl of lysis buffer were added. The tubes were immediately vortexed for 1 min, and incubated at 56°C on a shaker heater at 120 rpm overnight. Four hundred microliters of pure ethanol was added, and the mixture was transferred into spin columns. After centrifugation at 8,000 rpm for 1 min, the spin columns were transferred into new collection tubes and 500 µl of washing buffer 1 was added to each column. After centrifugation at 8,000 rpm for 1 min, the flowthrough was discarded and 500 µl of washing buffer 2 was added. The columns were again centrifuged at 12,000 rpm for 2 min and then transferred into new sterile 1.5 ml collection tubes, to which 150 µl of elution buffer was added. After 10 min of incubation at room temperature, the columns were centrifuged at 8,000 rpm for 1 min and then discarded. The concentration of extracted DNA and the protein was determined by spectrophotometry at 260 nm and 280 nm respectively and the ratio was calculated for purity. One hundred nanogram of total extracted DNA was used for PCR amplifications.

#### HPV plasmid DNA and PCR primers

HPV DNA-containing plasmids were provided by Bioneer, Geumcheon-Gu, Seoul, Korea the same provider of the PCR primers. Table 1 gives the sequences of the general and type-specific primers used in the study.

#### Nested PCR procedures

Detection of HPV DNA was first carried out by PCR amplification of HPV L1 region. To increase the sensitivity of HPV detection, nested PCRs were performed using MY09-MY11 as outer and GP5+-GP6+ as inner primers. Two microliters of the MY09-MY11 PCR product was used as template for the nested PCR amplification with GP5+-GP6+ primers.

HPV detection with primers MY09-MY11 was performed as described by the authors elsewhere (18,20,21). In brief, PCRs were performed in a final volume of 50 µl. Each PCR mixture contained 40 mM KCl, 10 mM Tris-HCl (pH 9), 1.5 mM MgCl<sub>2</sub>, a 200 µM concentration of each deoxynucleoside triphosphate (dNTP), 2.5 U of AmpliTaq Gold DNA polymerase (AccuPower® PCR Premix, Bioneer, Seoul, Korea, Cat#K-2011), 50 pmol of primers MY09 and MY11. Amplifications were performed with the following cycling profile: AmpliTaq Gold activation was performed by incubation at 94°C for 10 min followed by 40 cycles of 1 min denaturation at 94°C, 1 min

annealing at 55°C, and 1 min elongation at 72°C. The last cycle was followed by a final extension step of 7 min at 72°C.

PCRs with primers GP5+-GP6+ were performed as described in the other studies (18,22). In brief, PCRs were carried out in a final volume of 50 µl containing 40 mM KCl, 10 mM Tris-HCl (pH 9), 1.5 mM MgCl<sub>2</sub>, a 250 µM concentration of each dNTP, 2.5 U of AmpliTaq Gold DNA polymerase (AccuPower® PCR Premix, Bioneer, Seoul, Korea, Cat#K-2011), and 50 pmol of primers GP5+ and GP6+. The thermal cycling conditions were as follows: a 10-min activation step for the AmpliTaq Gold polymerase was followed by 40 cycles of 1-min denaturation at 94°C, 2-min annealing at 40°C, and 1.5-min elongation at 72°C. The last cycle was followed by a final 7-min extension step at 72°C (instead of 4 min). Internal control primers of the human β globin primers, GH20 and PC04 were amplified in negative samples to verify that there were no inhibitory elements present in the extracted DNA. If inhibition was observed, the sample was excluded from the study and another sample replaced.

Nested amplification of the GP-E6/E7 PCR products with type-specific primers was chosen to achieve exact typing of the HPV infections. Primers for the identification of high-risk genotypes 16, 18, 31, 33 were synthesized. The identification of each HPV type present was achieved by determining the size of the

nested PCR amplification product by gel electrophoresis. Table 1 gives the sequences of the type-specific primers used in the study and their final product size. The conditions for PCRs with E6 consensus primers were adapted from other study (18). In brief the protocol was 94°C for 1 min, 40°C for 1 min, and 72°C for 2 min for a total of 40 amplification cycles. The first cycle was preceded by a 4-min denaturation step at 94°C. The last cycle was followed by an additional 10-min elongation step at 72°C. PCRs with type-specific primers were performed under the following conditions: 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s. The first cycle was preceded by a 4-min denaturation step and the last cycle was followed by a 4-min elongation step (18). All PCRs were performed in a final volume of 50 µl containing 40 mM KCl, 10 mM Tris-HCl pH 9, a 250 µM concentration of each dNTP, 1.5 mM MgCl<sub>2</sub>, 2 U of TaqDNA polymerase (AccuPower® PCR Premix, Bioneer, Seoul, Korea, Cat#K-2011), and 33 pmol of each primer. Two microliters of the GPE6/E7 PCR product served as template for the nested PCRs.

All amplifications in this study were performed in one thermal cycler (Primus 96 advanced® Gradient, Peqlab Biotechnology GmbH, Erlangen, Germany). Seven microliters of the amplification products were analyzed by electrophoresis on 2% agarose gels and ethidium bromide staining.

**Table 1.** Sequences of the general and type specific primers used for PCR tests

HPV Primer	Sequence	Amplicon (bp)
MY09	5'-GCM CAG GGW CAT AAY AAT TGG-3'	450
MY11	5'-CGT CCM ARR GGA WAC TG-3'	
GP5+	5'-TTT GTT ACT GTG GTA GAT ACT AC-3'	150
GP6+	5'-GAA AAA TAA ACT GTA AAT CAT ATT C-3'	
GP-E6-3F	5'-GGG WGK KAC TGA AAT CGG T-3'	630
GP-E6-5B	5'-CTG AGC TGT CAR NTA ATT GCT CA-3'	
GP-E6-6B	5'-TCC TCT GAG TYG YCT AAT TGC TC-3'	
HPV 16F	5'-CAC AGT TAT GCA CAG AGC TGC-3'	457
HPV 16R	5'-CAT ATA TTC ATG CAA TGT AGG TGT A-3'	
HPV 18F	5'-CAC TTC ACT GCA AGA CAT AGA-3'	322
HPV 18R	5'-GTT GTG AAA TCG TCG TTT TTC A-3'	
HPV 31F	5'-GAA ATT GCA TGA ACT AAG CTC G-3'	263
HPV 31R	5'-CAC ATA TAC CTT TGT TTG TCA A-3'	
HPV 33F	5'-ACT ATA CAC AAC ATT GAA CTA-3'	398
HPV 33R	5'-GTT TTT ACA CGT CAC AGT GCA-3'	

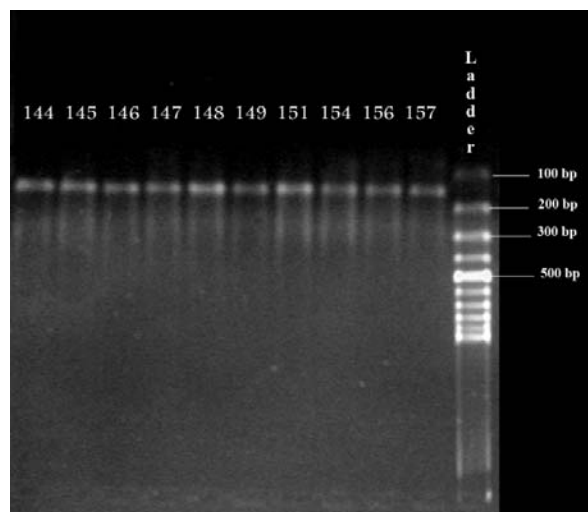
### Alternative PCR tests

For a greater degree of confidence, we used an alternative set of primers (HPVpU-1M/HPVpU-2R) and HPV-TM control positive template (PCR HPV typing kit, Takara Bio INC., Shiga, Japan, Cat#6603) to verify our results. HPV type specific detection carried out as it was described in the kit manual. In brief, PCRs were performed in a final volume of 50 µl. Each PCR mixture contained 40 mM KCl, 10 mM Tris-HCl (pH 9), 1.5 mM MgCl<sub>2</sub>, a 200 µM concentration of each deoxynucleoside triphosphate (dNTP), 2.5 U of AmpliTaq Gold DNA polymerase (AccuPower® PCR Premix, Bioneer, Seoul, Korea, Cat#K-2011), 0.5 µl of primers HPVpU-1M and HPVpU-2R and 100 ng of sample genome DNA. Amplifications were performed with the following cycling profile: AmpliTaq Gold activation was performed by incubation at 94 C for 5 min followed by 30 cycles of 30-sec denaturation at 94°C, 2-min annealing at 55°C, and 1-min elongation at 72°C. The last cycle was followed by a final extension step of 7 min at 72°C.

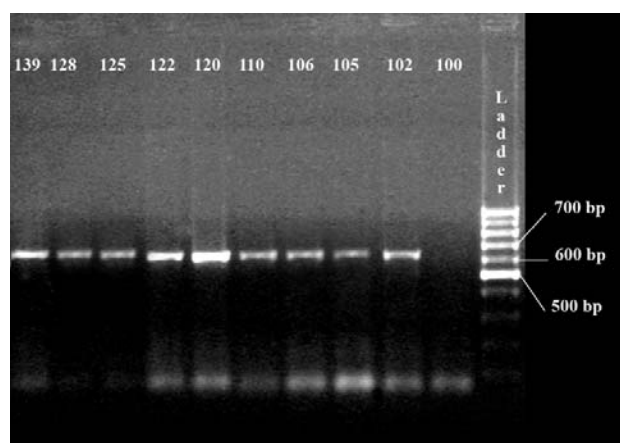
### Results

A total of 100 conjunctival tissue specimens diagnosed in Farabi Eye Hospital in the period from 2000 to 2006 were selected. Specimens were from 50 patients with squamous cell carcinoma (32 males and 18 females, aged 10 to 90 years) and 50 patients with normal lesion-free biopsies of the conjunctiva (29 men and 21 women, aged 9 to 84 years). HPV DNA was detected in 46 of 50 samples of squamous cell carcinoma and none of the normal biopsies by nested PCR using primer sets of the HPV consensus L1 region (MY/GP). The HPV DNA detected in the positive samples yielded strong signals (Figure 1). Subsequently, specimens from the 46 positive cases were subjected to specific PCR. Although 630bp amplicon was produced in 44 of 46 samples using consensus E6/E7 primers (Figure 2), none of the specific HPV PCR reactions for HPV DNA type 16, 18, 31 or 33 resulted in the detection of HPV DNA in the 44 SCC specimens of the conjunctiva. Fragments of human beta-actin gene that served as internal controls were amplified successfully in all 44 specimens.

In alternative tests mentioned in methods; the primers were set to detect malignant HPV types (16,18,31,33,35,52b,58). Again we were unable to detect any malignant HPV DNA fragments of specific types in all Specimens but HPV-TM control positive template.



**Figure 1.** HPV detection by MY/GP nested PCR (150 bp). Electrophoresis of PCR products on 2% agarose gel. The results of 10 samples are shown.



**Figure 2.** The PCR products generated with GP-E6/E7 primers (630 bp). Electrophoresis of PCR products on 2% agarose gel. The results of 10 samples are shown

### Discussion

This investigation was performed to determine the presence of HPV in conjunctival squamous cell carcinomas. HPVs are epitheliotropic oncogenic viruses. Most of them are found in benign epithelial proliferations and, therefore, are classified as low-risk HPV genotypes (the most common of these are HPV6 and HPV11), whereas certain viral genotypes are frequently found to be associated with malignant tumors and, thus, are considered as high-risk HPVs (the most common of these are HPV16, 18, 31 and 33). HPV DNA has been shown to induce malignant transformation of cultured cells *in vitro* (23).

**Table 2.** Previous investigations of conjunctival squamous cell carcinoma for the presence of human papillomavirus

Study	Year	Cases (Positive/Total)	HPV		Method
			Frequency (%)	HPV Type	
McDonnell <i>et al.</i> (34)	1989	10/10	100	16	PCR
Saegusa <i>et al.</i> (31)	1995	1/4	25	16	PCR/ISH
Tulvatana <i>et al.</i> (51)	2003	0/30	0	---	PCR/ISH
Ateenyi-Agaba <i>et al.</i> (52)	2004	18/21	86	12, 37, 8, 24, 14, 36, 38	PCR
Moubayed <i>et al.</i> (12)	2004	12/13	92	6/11, 16, 18	ImmunoMax ISH
Tornesello <i>et al.</i> (53)	2006	17/86	20	6, 18, Multiple Cutaneous HPV Types	Broad Spectrum PCR
De Koning <i>et al.</i> (47)	2008	11/29	38	14, 8, 17, 23	PCR
Asadi-Amoli <i>et al.</i> (current study)	2008	44/50	88	No type found, 16, 18, 31 and 33 rolled out	Nested PCR

The ability to transform cultured cells has been traced to E6 and E7 early open reading frames of the HPV genome (24). The E6-encoded transforming proteins confer tumorigenicity. The E7-encoded proteins confer malignant growth properties. There is evidence that E6- and E7-transforming proteins interact with nuclear protein (25). By using different molecular approaches, the presence of HPV DNA was detected in the SCC of several organs (26). However, the important etiologic role of HPV infection has been firmly established only in the development of anogenital SCC (7,27,28).

Conjunctival squamous neoplasia is classically referred to as being of actinic origin (26). The theory that UV light has a role in the development of these lesions is based on their position in the interpalpebral area and their predilection for older men, who are presumed to have heavier exposure to sunlight than women. HPV DNA has been identified within dysplastic lesions, including carcinomas of the conjunctiva (29-42). As summarized in table 2, HPV DNA has been searched for in conjunctival tissues by various molecular techniques. Owing to different populations studied and different HPV detection techniques, there are marked variations in the obtained HPV prevalence rates in conjunctival carcinoma, ranging from 0% to 100%. Today, the diagnosis of HPV infections is mostly based on the detection of viral DNA. Highly conserved regions in different parts of the viral genome have enabled the development of general or consensus PCR primer sets, such as MY09-MY11, PGMY09-MY11, GP5+-GP6+, SPF10, and LCR-E7 (43-46), which allow the detection of a broad spectrum of different HPV genotypes. However, differences in malignant potential mean that it is particularly important

to accurately identify infections with the high-risk HPV genotypes.

Based on our results, HPV is strongly probable to acts in the development of conjunctival SCC and the lack of HPV 16, 18, 31 and 33 in conjunctival SCC in this study may indicate that these HPV types are not associated with this condition. In a recent study (47), De Koning *et al.* found genital types of HPV in both case and control group in an equal rate (38% in cases and 38% in controls, OR=1) but cutaneous types of HPV in different rates (22% in cases vs 3% in controls). So they proved that there is no association between genital HPV types and SCC. We think the detection of genital HPV types in both cases and control group in their study could be attributable to high HIV seropositivity among their samples (64% of cases and 34% of controls).

In conclusion, our study presents a large amount of conjunctival squamous cell carcinoma investigated for HPV. It shows that HPV is strongly associated with conjunctival SCC. Furthermore, we found that unlike the genital mucosa (48), all normal conjunctival biopsy specimens were HPV negative showing that the conjunctiva is not an HPV reservoir in normal HIV seronegative patients. This is in accordance with the outcome of similar studies on the neighboring nasal and oral cavity, where HPV is likewise not present in normal mucosa (49,50).

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