



# Detection of a large spectrum of viral infections in conjunctival premalignant and malignant lesions

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## Abstract

To study the interaction between HIV and other carcinogenic infections in conjunctival squamous cell carcinoma (SCC), we evaluated the presence of a broad spectrum of human viruses in conjunctiva specimens. Beta Human papillomavirus (HPV; n = 46), gamma HPV (n = 52), polyomaviruses (n = 12) and herpes viruses (n = 3) was determined in DNA extracted from 67 neoplastic and 55 non-neoplastic conjunctival tissues of HIV-positive and HIV negative subjects by Luminex-based assays. Next-generation sequencing (NGS) was also used to further characterize the presence of cutaneous HPVs. Detection of beta-2 HPV infections was associated with the risk of neoplasia (adjusted odds ratio [aOR] 3.0; 95% confidence interval [CI] 1.3-6.8), regardless of HIV status (HIV positive, aOR 2.6, 95% CI 0.9-7.7; HIV negative, aOR 3.5, 95% CI 0.9-14.4). EBV was strongly associated with the risk of neoplasia (aOR 12.0, 95% CI 4.3-33.5;  $P < .01$ ) mainly in HIV individuals (HIV positive, aOR 57.5; 95% CI: 10.1-327.1; HIV negative aOR 2.6; 95% CI: 0.2-34.7). NGS allowed to identify 13 putative novel HPVs in cases and controls. Our findings suggest a role of beta HPV types and EBV, in conjunctival SCC. However, additional studies of viral expression in tumor tissue are required to confirm the causal association.

## KEYWORDS

conjunctiva SCC, EBV, HPV, next-generation sequencing

## 1 | INTRODUCTION

Squamous cell carcinoma (SCC) of the conjunctiva is a malignant disease of the eyes grouped in ocular surface squamous neoplasia (OSSN).<sup>1</sup> OSSN includes different types of lesion with various degrees

of dysplasia, carcinoma in situ and invasive squamous cell carcinoma of cornea and conjunctiva.<sup>2</sup> Incidence of conjunctival SCC has a large geographic variability, with high incidence rates occurring in Africa (about 1.3 per 100 000 person-years), while it is considered rare in other regions of the world (about 0.1 per 100 000 person-years).<sup>3,4</sup> Ultraviolet (UV) radiation represents the most important etiological factor of conjunctival SCC.<sup>5</sup> The impairment of the immune system also appears to contribute to conjunctival SCC development.<sup>6</sup> Indeed, HIV-positive individuals have nearly 10-fold increased risk of development of conjunctival SCC in comparison to HIV-negative population.<sup>7</sup> Accordingly, this pathological condition has greatly increased in

**Abbreviations:** aOR, adjusted odds ratio; CI, confidence interval; cSCC, conjunctival squamous cell carcinoma; CIN, conjunctival intraepithelial neoplasia; HPV, human papillomavirus; NGS, next-generation sequencing; OSSN, ocular surface squamous neoplasia; RAxML, Randomized Axelerated Maximum Likelihood.

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several African countries in the last decades, coinciding with the spread of HIV infection.<sup>8,9</sup> In addition, an increased incidence rate of conjunctival SCC has been observed in Australian subtropical area in immune-compromised individuals after renal transplantation.<sup>10</sup> The apparent link of the immune suppression and development of conjunctival SCC strongly suggests the involvement of infectious agents in the etiology of this type of cancer. Several studies showed that cutaneous beta-HPV types were more present in conjunctival SCC rather than other nonmalignant eye conditions.<sup>11-13</sup> A few studies also suggested the involvement of mucosal high-risk alpha HPV types in the conjunctival SCC development, however other studies did not support this association.<sup>14-18</sup> Finally, some studies investigated the role of additional human viruses, such as Epstein-Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV), Herpes Simplex Virus (HSV) and Cytomegalovirus (CMV) in both OSSN and UV-related noncancerous eye conditions, as pterygium.<sup>6,19,20</sup> Thus, the etiological role of an infectious agent in conjunctival SCC remains a matter of debate and deserves more investigation.

In our study, we have evaluated the presence of a broad spectrum of cutaneous human papillomaviruses, herpesviruses and polyomaviruses using highly sensitive Luminex-based assays in conjunctival samples. The study included HIV-positive and HIV-negative cancer free-subjects as well as those with precancerous and cancerous lesions recruited in a previous case-control study.<sup>21</sup> In addition, the Luminex assay was complemented by the use of a next-generation sequencing (NGS)-based strategy to further evaluate the possible presence of additional novel or already characterized cutaneous HPV types.

## 2 | MATERIALS AND METHODS

### 2.1 | Study population and biological specimens

We have analyzed total DNA extracted from conjunctival tissues (n = 67 cases) including conjunctival intraepithelial neoplasia (CIN 1 = 15; CIN 2 = 15, CIN3 = 15), and conjunctiva squamous cell carcinoma SCC (n = 22) as described in a previously published study.<sup>21</sup> Conjunctiva non-neoplastic biopsies (n = 55 controls) obtained from the same study were also analyzed.<sup>21</sup> Briefly, subjects with conjunctiva neoplasia were enrolled in seven countrywide eye clinics in Southern Uganda between January 1997 and March 1999. In the same clinics, tissue specimens were collected also for control subjects that were randomly enrolled among patients presenting benign lesion (eg, pterygium) or eye injuries. Peripheral whole blood samples were obtained from all case patients and control subjects at surgical removal of conjunctival lesions and serum tested for anti-HIV1/2 antibodies by enzyme-linked immunosorbent assay (ELISA). Positive samples were further tested by immunoblotting. All enrolled subjects did not receive antiretroviral treatments, since they were adopted in Uganda in 2004 after the collection of the specimens used in our study. No information on the CD4 lymphocyte count was available. However, the majority of HIV-positive patients and controls were

### What's new?

Risk of conjunctival squamous cell carcinoma (SCC) is strongly associated with ultraviolet radiation exposure and immune suppression. In Africa, SCC incidence has also been affected by the spread of HIV and may be impacted by other viral infections. Here, analyses of a large number of viruses in conjunctival non-malignant and malignant tissues from patients in Southern Uganda reveal associations between conjunctival SCC and cutaneous beta human papillomavirus (HPV) and Epstein-Barr virus (EBV) infection. Beta-2 HPV infection was linked to neoplasia risk regardless of HIV status, whereas EBV was associated with neoplasia primarily in HIV-infected individuals.

classified as WHO clinical Stage 1 and only three cases classified as WHO clinical Stage 2.

Total DNA extracted from specimens of cases (n = 86) and controls (n = 63) were previously analyzed for the presence of alpha and beta HPVs.<sup>21</sup> Part of these samples was further analyzed for HHV8 infection (72 cases and 60 controls)<sup>20</sup> as well as for TERT promoter mutations (67 cases and 55 controls).<sup>34</sup> In the present study, we have analyzed the samples that remained available after the completion of the previous studies. Total nucleic acids were extracted in Naples as previously described<sup>21</sup> and were analyzed by Luminex and NGS at IARC (Lyon, France).

### 2.2 | Detection of viral DNA by Luminex assay and statistical analysis

The prevalence of several infectious agents was determined by using type-specific multiplex genotyping (TS-MPG) assays, which combine multiplex polymerase chain reaction (PCR) and bead-based Luminex technology (Luminex Corp., Austin, Texas), as described previously.<sup>22-26</sup> Multiplex PCR amplifications using type-specific primers were used for the detection of 46 genus beta-HPV types (HPV5, HPV8, HPV9, HPV12, HPV14, HPV15, HPV17, HPV19, HPV20, HPV21, HPV22, HPV23, HPV24, HPV25, HPV36, HPV37, HPV38, HPV47, HPV49, HPV75, HPV76, HPV80, HPV92, HPV93, HPV96, HPV98, HPV99, HPV100, HPV104, HPV105, HPV107, HPV110, HPV111, HPV113, HPV115, HPV118, HPV120, HPV122, HPV124, HPV143, HPV145, HPV150, HPV151, HPV152, HPV159 and HPV174), 52 genus gamma-HPVs (HPV4, HPV48, HPV50, HPV60, HPV65, HPV88, HPV95, HPV101, HPV103, HPV108, HPV109, HPV112, HPV116, HPV119, HPV121, HPV123, HPV126, HPV127, HPV128, HPV129, HPV130, HPV131, HPV132, HPV133, HPV134, HPV148, HPV149, HPV156, HPV161, HPV162, HPV163, HPV164, HPV165, HPV166, HPV167, HPV168, HPV169, HPV170, HPV171, HPV172, HPV173, HPV175, HPV178, HPV179, HPV180, HPV184, HPV197, HPV199, HPV200, HPV201, HPV202 and SD2), 12 polyomaviruses (BKV, JCV, KIV, WUV, MCPyV, HPyV6, HPyV7, TSV, HPyV9, HPyV10, HPyV12

and LIPyV) and three herpesviruses (CMV, EBV1 and EBV2). Two primers for the PCR amplification of beta-globin gene were also used to assess the quality of the extracted DNA. After PCR amplification, 10  $\mu$ L of each reaction was analyzed by multiplex using a Luminex-based assay as described in detail previously.<sup>25,27</sup>

Prevalence of infectious agents is given as a percentage and corresponding 95% confidence intervals (95% CI) were calculated according to the binomial distribution. Odds ratios (ORs) and 95% CIs were calculated using unconditional logistic regression models and were adjusted for age and sex.

## 2.3 | Next-generation sequencing procedures

Extracted DNA was amplified using two different sets of primers (FAP59 \FAP64 and FAPM1 primer mix) as previously described.<sup>28,29</sup> Both FAP and FAPM1 primer sets target a region of the L1 ORF yielding an amplicon of about 480 bp. PCR amplicons were visualized by electrophoresis on a 1.5% agarose gel and purified using QIAquick gel extraction purification kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). Purified PCR amplicons of the expected size were divided into four different pools using the same volume of each purified amplicons, according to the PCR protocol and conjunctiva specimens as reported in Table 1.

Before library preparation, one additional purification step was performed in each pool to remove any residual contaminants using the Agencourt AMPure XP PCR purification kit with a beads ratio of 1.8 X (Beckman Coulter) according to the manufacturer's instructions. The amplicon-based libraries were prepared using the Nextera DNA Flex Library preparation kit (Illumina, San Diego, California). Illumina MiSeq dual-indexed adapters (Illumina, San Diego, California) were added to each of the PCR pools. The library sizes were checked using the ScreenTape System (Agilent) using high sensitivity assay. NGS analysis was performed on 4 nM of DNA pooled library using the Illumina benchtop MiSeq sequencer (2  $\times$  150 paired-end reads with the Illumina MiSeq kit v3). To enrich the diversity of the libraries, 10% of PhiX (Illumina, San Diego, California) was added to the NGS reaction.

## 2.4 | Bioinformatic analysis

All the bioinformatic analyses were performed using PVAmpliconFinder (<https://github.com/IARcbioinfo/PVAmpliconFinder>), and the main steps

**TABLE 1** Description of the NGS pools stratified according to conjunctiva samples and PCR protocol

NGS pool	PCR primers	Sample group	Total number
1	FAP59-FAP64	Conjunctiva casesa	33
2	FAPM1	Conjunctiva casesa	17
3	FAP59-FAP64	Conjunctiva controls	24
4	FAPM1	Conjunctiva controls	43

Note: Each pool is constituted of PCR products that gave an amplicon of about 500 bp with the indicated primers.

<sup>a</sup>Cases group includes conjunctiva papilloma (n = 1).

are described as follow. The raw data were first preprocessed for quality control and filtering. Several steps of data complexity reduction were applied, and DNA sequences were subsequently identified based on similarity when compared against available PVs sequences in the NCBI database. DNA sequences were assigned as a known HPV type when it showed a high identity of more than  $\geq 90\%$  with L1 ORF of the closest HPV type. A putative novel HPV sequence was assigned as a putative new HPV type when reported a low identity of less than  $< 90\%$  with L1 ORF of the closest PV. De-novo reconstruction of the fully amplified region covered by several primers systems was performed. Finally, the reconstructed sequences were taxonomically classified by both alignment-based and homology-based methodologies. All the results in our study are based on the homology-based classification using the evolutionary placement algorithm (EPA) in RAxML (Randomized Axelerated Maximum Likelihood).<sup>29,30</sup> Only the longest sequence was considered for the RAxML-EPA classification when several singlets or contigs were available.

## 3 | RESULTS

### 3.1 | Detection of viral DNA by Luminex assay

We analyzed the neoplasia cases (n = 67) included different grades of conjunctival intraepithelial neoplasia (CIN 1, n = 15; CIN 2, n = 15, CIN3, n = 15), and conjunctiva squamous cell carcinoma cSCC (n = 22), and the cancer-free controls (n = 55) for the presence of cutaneous beta and gamma HPV types, polyomaviruses and herpesviruses by Luminex-based assays. The median age of the study population was 32 years (IQR, 27-40), of them 49.2% (n = 60) were female while 50.8% (n = 62) were male (Table 2). The proportion of neoplastic lesions was significantly higher in females (59.7%) compared to males (40.3%;  $P = .01$ ; Table 2). In the neoplasia and control group, 64.2% (n = 43/67) and 54.5% (n = 30/55), respectively, were positive for HIV. The samples were all positive for beta-globin gene thus being valid for the analysis. Beta HPV types were found in 45.4% (n = 25/55) and 61.2% (n = 41/67) of controls and neoplasia group, respectively, but the difference was not statistically significant. However, the stratification of the beta HPV types into the different species revealed a significantly higher beta-2 HPV prevalence in cases ( $P < .01$ ; Table 2 and Figure 1). Such association remained significant also after adjustment for age, and sex (aOR 3.0; 95% CI: 1.3-6.8; Table 3). When stratified by HIV status, no clear difference in beta 2-positivity between HIV-positive (aOR 2.6; 95% CI: 0.9-7.7) and HIV-negative subjects (aOR 3.5; 95% CI: 0.9-14.4) was found (Table 4). On the contrary, there was no statistically significant association between neoplasia and beta-1 HPV types ( $P = .25$ ; Tables 2 and 3 and Figure 1). The most prevalent HPV types in beta-1 species were HPV5 (n = 15) and 24 (n = 8), in beta-2 species were HPV38 (n = 21) and HPV100 (n = 8), and in beta-3 species was HPV75 (n = 12). However, there was a significant difference between cases and controls only for HPV5 (aOR 9.1; 95% CI: 1.1-75.0;  $P = .04$ ; data not shown).

**TABLE 2** Characteristics of the study population

	All (122) n (%)	Controls <sup>a</sup> (55) n (%)	Cases (67) n (%)	p <sup>b</sup>
Age (median [IQR])	32 (27-40)	32 (26-40)	32 (27-40)	
<30	41 (33.6)	17 (30.9)	24 (35.8)	
30 to 39	47 (38.5)	21 (38.2)	26 (38.8)	
40+	34 (27.9)	17 (30.9)	17 (25.4)	.47
Sex				
Female	60 (49.2)	20 (36.4)	40 (59.7)	
Male	62 (50.8)	35 (63.6)	27 (40.3)	.01
HIV status				
Negative	49 (40.2)	25 (45.5)	24 (35.8)	
Positive	73 (59.8)	30 (54.5)	43 (64.2)	.35
EBV1 and EBV2				
Negative	75 (61.5)	49 (89.1)	26 (38.8)	
Positive	47 (38.5)	6 (10.9)	41 (61.2)	<.01
EBV1				
Negative	87 (71.3)	49 (89.1)	38 (56.7)	
Positive	35 (28.7)	6 (10.9)	29 (43.3)	<.01
EBV2				
Negative	93 (76.2)	52 (94.5)	41 (61.2)	
Positive	29 (23.8)	3 (5.5)	26 (38.8)	<.01
MCPyV				
Negative	79 (64.8)	40 (72.7)	39 (58.2)	
Positive	43 (35.3)	15 (27.3)	28 (41.8)	.13
β-HPV [46]				
Negative	56 (45.9)	30 (54.5)	26 (38.8)	
Positive	66 (54.1)	25 (45.5)	41 (61.2)	.10
β1-HPV [19]				
Negative	82 (67.2)	40 (72.7)	42 (62.7)	
Positive	40 (32.8)	15 (27.3)	25 (37.3)	.25
β2-HPV [20]				
Negative	77 (63.1)	42 (76.4)	35 (52.2)	
Positive	45 (36.9)	13 (23.6)	32 (47.8)	<.01
β3-HPV [4]				
Negative	108 (88.5)	49 (89.1)	59 (88.1)	
Positive	14 (11.5)	6 (10.9)	8 (11.9)	.86
γ-HPV [52]				
Negative	105 (86.1)	49 (89.1)	56 (83.6)	
Positive	17 (13.9)	6 (10.9)	11 (16.4)	.48

<sup>a</sup>Controls includes conjunctiva papilloma, pinguecula and inflammation (n = 4).

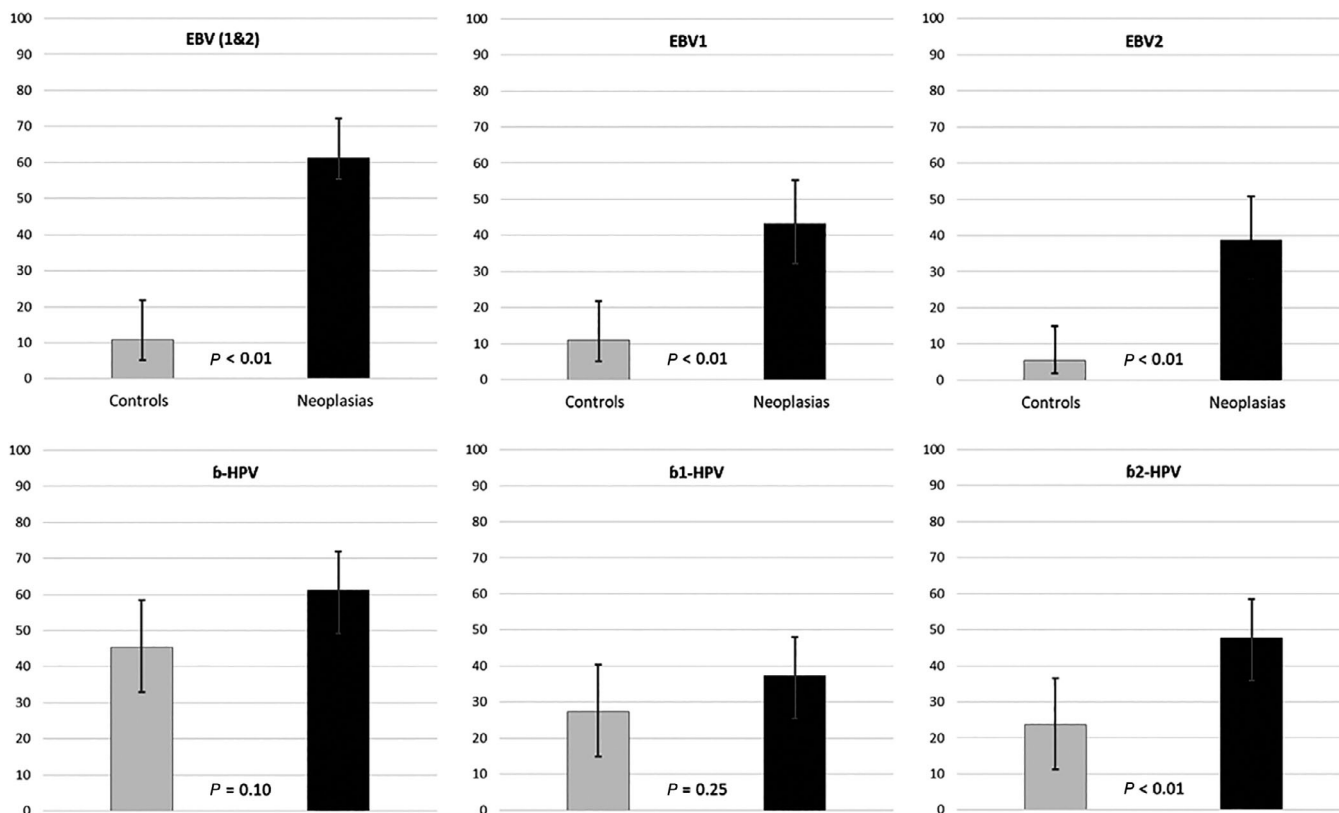
<sup>b</sup>Pearson's Chi-square test.

The remaining HPV types that belong to beta species 3 to 5 were less abundantly detected (beta-3) (Table 2) or absent (beta 4-5) in both groups.

Gamma HPVs were detected in 13.9% (n = 17/122). Of these 10.9% (n = 6/55) were in controls and 16.4% (n = 11/67) in cases, respectively. Regarding gamma-HPV types, no significant differences were found between cases and controls (P = 0.48; Table 2).

Regarding the polyomaviruses (HPyVs), the most prevalent was MCPyV (35.3%) as shown in Table 2. However, no significant difference in prevalence between cases and control was found (P = .13). Some of the remaining PyVs tested were rarely detected or absent (data not shown).

Regarding the tested herpes viruses, CMV was detected in 5.4% (n = 3) of controls and in 8.9% of cases (n = 6), while EBV1 and 2 were



**FIGURE 1** Prevalence of indicated viruses and corresponding 95% confidence intervals (CI) in controls and neoplasia groups

**TABLE 3** Odds ratio for positivity of EBV1&EBV2, β1-HPV and β2-HPV and corresponding 95% confidence Interval

	Controls <sup>a</sup> (55) n (%)	Cases (67) n (%)	OR [95% CI]	P	aOR <sup>a</sup> [95% CI]	P
<b>EBV1 &amp; EBV2</b>						
Negative	49 (89.1)	26 (38.8)	Ref		Ref	
Positive	6 (10.9)	41 (61.2)	12.6 [4.8-34.3]	<.01	12.0 [4.3-33.5]	<.01
<b>β1-HPV</b>						
Negative	40 (72.7)	42 (62.7)	Ref		Ref	
Positive	15 (27.3)	25 (37.3)	1.6 [0.7-3.4]	.24	1.3 [0.6-2.9]	.54
<b>β2-HPV</b>						
Negative	42 (76.4)	35 (52.2)	Ref		Ref	
Positive	13 (23.6)	32 (47.8)	3.0 [1.3-6.5]	<.01	3.0 [1.3-6.8]	.01

<sup>a</sup>Adjusted on age and sex.

abundantly detected in both groups (38.5%) as shown in Table 2 and Figure 1. However, EBV prevalence was much higher in the neoplasia group than controls, 61.2% vs 10.9%, respectively ( $P = <.01$ ) (Table 2 and Figure 1). EBV remained significantly associated also after adjustment for age, and sex (aOR 12.0; 95% CI: 4.3-33.5; Table 3). Prevalence of EBV1 and EBV2 was significantly higher in the neoplastic group ( $P < .01$ ) than the controls (Table 2). Stratified by HIV status, the EBV-positivity was strongly associated with cases (aOR 57.5; 95% CI: 10.1-327.1) in HIV-positive individuals. On the contrary, the EBV-positivity was not associated with cases in HIV-negative individuals (aOR 2.6; 95% CI: 0.2-34.7;  $P = .47$ ; Table 4).

Of note, by severity of histological lesion, prevalence of EBV in HIV-positive individuals was 81.8%, 100% and 100% for CIN1, CIN2 and CIN3, respectively and 85.7% in SCC, being significantly higher than the control ( $P$  for trend  $< .01$ ; Table 5).

### 3.2 | HPVs broad-spectrum analysis by NGS

To have a broader view on the distribution of HPV types in cases and controls we have performed NGS analysis. A total of 2 415 171 paired-end raw reads was obtained for the NGS analysis. After quality

**TABLE 4** Odds ratio for positivity of EBV1&EBV2 and  $\beta$ 2-HPV and corresponding 95% confidence interval by HIV status

	Controls <sup>a</sup> (55) n (%)	Cases (67) n (%)	OR [95% CI]	P	aOR <sup>b</sup> [95% CI]	P
HIV positive						
EBV1&EBV2 negative	25 (83.3)	4 (9.3)	Ref		Ref	
EBV1&EBV2 positive	5 (16.7)	39 (90.7)	48.8 [11.9-199.2]	<.01	57.5 [10.1-327.1]	<.01
HIV negative						
EBV1&2 negative	24 (96.0)	22 (91.7)	Ref		Ref	
EBV1&2 positive	1 (4.0)	2 (8.3)	2.2 [0.2-25.8]	.54	2.6 [0.2-34.7]	.47
HIV positive						
$\beta$ 2-HPV negative	21 (70.0)	20 (46.5)	Ref		Ref	
$\beta$ 2-HPV positive	9 (30.0)	23 (53.5)	2.7 [1.0-7.2]	<.05	2.6 [0.9-7.7]	.09
HIV negative						
$\beta$ 2-HPV negative	21 (84.0)	15 (62.5)	Ref		Ref	
$\beta$ 2-HPV positive	4 (16.0)	9 (37.5)	3.2 [0.8-12.2]	.10	3.5 [0.9-14.4]	.08

<sup>a</sup> Controls includes conjunctiva papilloma, pinguecula and inflammation (n=4).

<sup>b</sup> Adjusted on age and sex.

**TABLE 5** EBV positivity in controls and preneoplastic/neoplastic conjunctival lesions, by HIV status

	Controls n (%)	CIN 1 n (%)	CIN 2 n (%)	CIN 3 n (%)	SCC n (%)	P for trend
All participants						
EBV-negative	49 (89.1)	6 (40.0)	5 (33.3)	6 (40.0)	9 (40.9)	
EBV-positive	6 (10.9)	9 (60.0)	10 (66.7)	9 (60.0)	13 (59.1)	<.01
HIV negative						
EBV-negative	24 (96.0)	4 (100)	5 (100)	6 (85.7)	7 (87.5)	
EBV-positive	1 (4.0)	0 (0.0)	0 (0.0)	1 (14.3)	1 (12.5)	.28
HIV positive						
EBV-negative	25 (83.3)	2 (18.2)	0 (0.0)	0 (0.0)	2 (14.3)	
EBV-positive	5 (16.7)	9 (81.8)	10 (100)	8 (100)	12 (85.7)	<.01

trimming, de-replication, and chimeric PCR sequence removal, 2 548 514 reads were considered for further analysis. Approximately 58.7% (1 498 086 reads) were related to known HPVs and 0.03% (785 reads) to novel putative PVs. According to the similarity with available PV sequences in the NCBI database, a total of 207 known and 13 putative new HPV sequences were detected in our study (Tables S1 and S2).

Among the known HPV sequences the alpha-related reads (generated by both FAP and FAPM1 PCR protocols) in pools 1-2 (cases) vs 3-4 (controls) was of 502 602 (19%) and 75 reads (0.002%) respectively. The majority of the reads in cases pools were referred to HPV11 (200 868 and 298 441 reads obtained with FAP and FAPM1 primers, respectively).

A total of 685 432 reads (26.9%) were assigned to beta-HPV types; 366 593 (14.4%) reads were detected in cases while the control group generated 318 839 (12.5%) sequences. In detail, the number of reads related to beta-1 species was 266 367 in cases vs 249 437 in controls, while 64 338 reads in cases and 69 227 reads in controls were related to beta-2 species. The number of reads from beta-3 species was higher in cases (25 319 reads) than in controls (156 reads).

Beta-5 species (166 reads) reads were exclusively found in cases. As previously suggested by the Luminex analysis, no sequences from beta-4 species were found in both groups.

Specifically, the number of reads of the following HPV types HPV5 ( $\beta$ -1), 8 ( $\beta$ -1), HPV19 ( $\beta$ -1), HPV24 ( $\beta$ -1), HPV38 ( $\beta$ -2), HPV49 ( $\beta$ -3) and HPV75 ( $\beta$ -3) were exclusively detected or predominant in cases vs controls (Table S1). While the number of reads of other HPV types, for example, HPV12 ( $\beta$ -1), HPV14 ( $\beta$ -1), HPV23 ( $\beta$ -2) and HPV104 ( $\beta$ -2), had the opposite trend (Table S1). A small number of reads (10 403 reads in cases and 19 reads in controls) corresponded to beta unreferenced HPVs.

A total of 161 698 (6.3%) and 64 240 (2.5%) gamma-HPV types related sequences was detected in cases and controls, respectively, spreading into different gamma species or unreferenced-HPV gamma types (Table S1).

Finally, 785 reads (0.03%) allowed the identification of 13 putative novel PVs, as their sequences showed less than 90% similarity to L1 ORF of any known PVs. According to RaxML-EPA classification, the putative novel sequences were closely related to beta-1, beta-2 and gamma-1, 11, 15 and other unreferenced HPVs.

The majority of the putative novel PVs ( $n = 11$ ) were found in cases (Table S2).

## 4 | DISCUSSION

The association between UV with conjunctival SCC is well established.<sup>30,31</sup> In addition, impairment of the immune system by HIV, or in solid organ transplant recipients (SOTR), appears to be a marked risk factor for conjunctival SCC.<sup>7</sup> The fact that immune-system impairment increases the susceptibility to conjunctival SCC, strongly suggests the involvement of infectious agents in this disease. Several studies present evidence for the contribution of HPV to conjunctival SCC,<sup>6,19,20</sup> although this association remains controversial. Our study showed that beta-2 HPV infections was associated with the risk of neoplasia (aOR 3.0; 95% CI 1.3-6.8), as previously reported for cutaneous SCC.<sup>32</sup> Thus, the proposed scenario in conjunctival SCC appears to share similarities with the one proposed for the skin carcinogenesis, where UV radiation is the leading risk factor together with immunosuppression.<sup>33</sup> This similarity is further underlined by the mutation profile detected in both SCCs.<sup>34</sup> Solid organ transplant recipients (SOTR) have about 100-fold risk of cutaneous SCC in comparison to the healthy individuals.<sup>35</sup> Interestingly, a previous study reported that the incidence of conjunctival OSSN, including conjunctival SCC, was 20-fold increase in kidney transplantation subjects living in the Australian subtropical area.<sup>10</sup> Importantly, these OTRs had also history of cutaneous SCC, suggesting that both type of cancers share the same etiological factors.<sup>10</sup>

Our Luminex data showed that HPV5 and 24 from beta-1 species and HPV38 and HPV100 from beta-2 species were the most prevalent types in cases and controls. Among them, only HPV5 was the only significantly associated with cases (aOR 9.1; 95% CI: 1.1-75.0;  $P = .04$ ). HPV types 5, 24, 38 and 100 were previously detected in other case-control studies.<sup>11-13,21</sup> IARC has classified beta-1 species HPV5 and HPV8 as "possibly carcinogenic" agents (Group 2B) in skin lesions in *Epidermodysplasia verruciformis* patients.<sup>36</sup> Beta types such as HPV5, HPV12, HPV24 and HPV38 were also frequently reported in multiple anatomic sites.<sup>37</sup> Interestingly, beta-3 species HPV75 was frequently found in our study (overall prevalence 9.8%), while in other studies, this type was rarely found compared to the other beta-3 HPV types (eg, HPV49 and HPV76) at different anatomical sites.<sup>37,38</sup>

Interestingly, our results are also in line with other studies on cutaneous SCC. Using different viral markers, these studies reported a more significant association of beta-2 HPV types with risk of cutaneous SCC development in comparison to the other beta species.<sup>32,39,40</sup>

Our complementing strategy using NGS, revealed the presence of reads assigned to alpha-species in ocular tissues, confirming the previous findings from Tornesello et al.<sup>21</sup> We also observed a correlation between the Luminex-based prevalence study and the NGS data. Most of the HPV types were detected by both methodologies. In addition, by using two PCR protocols combined with NGS a total of 13 putative novel HPVs were identified. According to RaxML-EPA classification, the putative novel sequences were phylogenetically

related to HPV9, HPV20, HPV205, HPV213, HPV173, HPV169, HPV179 and unreferenced  $\gamma$ -HPVs (HPV-mw11C39 and HPV-mSK123). Eleven of them were found exclusively in cases. However, the NGS findings have the limitation due to the pool-based strategy, which does not allow the determination of prevalence of specific HPVs in case and controls.

Our Luminex results also show that EBV is significantly associated with cases (aOR 12.0; 95% CI: 4.3-33.5). Furthermore, the EBV-positivity was strongly associated with cases in HIV-positive individuals (aOR 57.5; 95% CI: 10.1-327.1), while the same result was not seen in HIV-negative (aOR 2.6 95% CI: 0.2-34.7;  $P = .47$ ). Our data support a role of EBV in the development of conjunctival SCC, as in other human cancers, such as nasopharyngeal SCC.<sup>7</sup> Moreover, the comparable trend of EBV prevalence in conjunctiva SCC and in conjunctival lesions (CIN1-CIN3) suggests that the virus is involved in early stage of neoplasia development all the way to tumor development. This suggests that EBV may be the, as yet undiscovered, carcinogenic agent that is expected to be working in synergy with HIV-related immunosuppression to cause conjunctiva SCC. Indeed, of all the cancer types established to be caused by HIV, conjunctiva SCC remains the only one for which there is not an established underlying infectious cause.<sup>7</sup>

However, our results cannot conclusively prove that EBV plays a direct role in the development of conjunctival SCC. It is also possible that EBV positivity in cancer tissue as well as in premalignant lesions of HIV-positive individuals is due to the presence of infiltrating viral-infected B lymphocytes.<sup>41</sup> To evaluate both possibilities additional analyses of conjunctiva SCC tissues are required, aiming to precisely determine the presence of different EBV markers (eg, EBNA 1 immunohistochemistry staining) in epithelial and/or B cells.

In conclusion, our findings reinforce the hypothesis that conjunctival SCC development is linked to viral infections. Whether EBV is present in cancer cells or in B-cell recruited at cancer site remain to be demonstrated. Importantly, it is essential to corroborate these data conducting similar studies in other geographical area with high conjunctival SCC incidence.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The datasets are available from the corresponding author on reasonable request.

## ETHICS STATEMENT

The study was approved by the Institutional Scientific Board of the Istituto Nazionale Tumori "Fondazione Pascale," Napoli, Italy. The informed consent was unavailable due to the retrospective design of the study and the large proportion of untraceable patients. Archived biological specimens were collected between January 1997 and March 1999, they were unequivocally identified and de-linked to ensure data protection, anonymization and patients privacy. Our study was performed in accordance with the Declaration of Helsinki.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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