1 **Title:** Human papillomavirus infection does not predict response to interferon
2 therapy in ocular surface squamous neoplasia

3 **Running Title:** HPV infection does not predict OSSN response to interferon

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Abstract

Purpose: To identify the frequency of human papilloma virus (HPV) in ocular surface squamous neoplasia (OSSN), and evaluate differences in clinical features and treatment response of HPV positive versus negative tumors.

Design: Retrospective case series.

Participants: 27 patients with OSSN

Methods: OSSN specimens were analyzed for the presence of HPV. Clinical features and response to interferon were retrospectively determined and linked to the presence (versus absence) of HPV.

Main Outcome Measures: Clinical characteristics of OSSN by HPV status.

Results: Twenty one of 27 tumors (78%) were HPV positive. HPV genotypes identified included HPV 16 in 10 (48%), HPV 31 in 5, HPV 33 in 1, HPV 35 in 2, HPV 51 in 2, and a novel HPV in 3 (total 23 as one tumor had 3 genotypes identified). Tumors found in the superior limbus were more likely to be HPV positive (48% vs 0%, Fisher exact $P = 0.06$). HPV 16 positive tumors were larger (68mm^2 vs 34mm^2 , Mann Whitney U $P = 0.08$) and were more likely to have a papillomatous morphology (50% vs 12%, Fisher exact $P = 0.07$) compared to HPV 16 negative tumors. HPV status was not found to associate with response to interferon therapy (Fisher exact $P = 1.0$). Metrics found to associate with a

45 non-favorable response to interferon were male gender and tumors located in the
46 superior conjunctivae.

47 **Conclusions:** HPV presence in OSSN appears more common in lesions located
48 in the non-exposed, superior limbus. HPV presence does not seem to be
49 required for a favorable response to interferon therapy.

50

Introduction:

Ocular surface squamous neoplasia (OSSN) represents a spectrum of disease, ranging from mild dysplasia to invasive squamous cell carcinoma (SCC), and is the most common non-pigmented tumor of the ocular surface.¹ While human papillomavirus has been implicated in the pathogenesis of a variety of cancers, most notably cervical cancer², its association with OSSN has been a subject of much debate. Our group previously found HPV DNA and mRNA in 10 out of 10 OSSN specimens (five HPV 16 and five HPV 18); neither HPV was detected in control specimens or in any clinically uninvolved conjunctival specimens from OSSN eyes.³ Other groups, however, have not replicated these findings with some studies reporting no HPV and others reporting lower rates of HPV detection in OSSN (Table 1).⁴⁻²⁰ It is not clear why certain areas seem to have a very low frequency of HPV in OSSN tumors (e.g. India, Germany, Taiwan)⁴⁻⁶, while others (e.g. Miami)³ have a much higher frequency. The effect of latitude and sexual activity on HPV positivity in OSSN is worth further study.

Understanding the epidemiology of HPV in OSSN is important since in non-ocular HPV associated malignancies, prognosis and treatment may be altered based on viral presence.² For example, some HPV positive tumors are treated with interferon (IFN) in isolation or as part of combination therapy.^{2,21} While its exact mechanism is unknown, interferon is known to have both anti-viral and anti-neoplastic properties.²² For OSSN, interferon has become a popular treatment modality and has been reported successful in 80–90% of OSSN tumors.^{23,24} Conversely, 10-20% of tumors do not respond to therapy. It is

currently unknown what tumor factors such as clinical features or viral presence might predict response or lack of efficacy to IFN. This information is important as it can help individualize therapy based on tumor characteristics. For example, physicians may proceed directly to surgery, or use a different agent, in patients in whom interferon is unlikely to be effective. The aim of this study was to evaluate the frequency of HPV in our more recent OSSN specimens, and to examine whether clinical characteristics, including response to interferon, were different based on HPV status.

Materials and Methods

Samples: Approval was obtained from the University of Miami Institutional Review Board and the methods adhered to the tenets of the Declaration of Helsinki and were HIPAA-compliant. Twenty-eight OSSN specimens, collected from 27 patients between 03/18/1997 and 2/14/2013, underwent testing for HPV presence. Patient records were retrospectively reviewed for information on demographics and prior OSSN history. Clinical features were also collected by chart review and photographs, when available. Clinical features studied included lesion location, clinical appearance (papillomatous, leukoplakic, gelatinous, flat/nodular); and size. Furthermore, tumors were staged based on the American Joint Committee on Cancer (AJCC) clinical staging system.²⁵

HPV testing: In situ hybridization was used to evaluate for HPV in OSSN tissue.^{26,27} In brief, multiple four micron sections were placed on sequentially labeled silane coated slides. The tissue was deparaffinized, proteased (30

minutes in 2mg/mL of pepsin), washed in sterile water, then 100% ethanol, and air dried. The probe cocktail containing the biotin-labeled genomic probe and the tissue DNA were co-denatured at 95°C for 5 minutes, hybridized for 15 hours at 37°C, then washed at either low (T_m 30°C, for identification of HPV DNA) or high stringency (T_m 58°C, for identification of the specific HPV type). Streptavidin conjugated alkaline phosphatase then reacted with the chromogen nitroblue tetrazolium and bromochloroindolyl phosphate (NBT/BCIP) to localize the probe/target complex. Nuclear fast red served as the counterstain. All samples were tested for HPVs 2, 6, 11, 13, 16, 18, 26, 27, 30, 31, 32, 33, 35, 39, 40, 41, 42, 43, 44, 45, 51, 52, 56, 59, 68, 70 as well as other “novel” types (an HPV detected that is related to but distinct from those included in the probe cocktail) as previously described.^{26,27} An HPV 16 positive cervical intra-epithelial neoplasia lesion and an HPV 6/11 positive genital condyloma lesion were utilized as positive controls. The negative controls were cervical and ocular squamous cell lesions that were histologically and molecularly negative for HPV infection.

p53 detection: The immunohistochemistry detection of p53 was done using our previously published protocol.²⁶ In brief, the optimal conditions for detection of p53 using the antibody from Enzo Life Sciences (Farmingdale, NY, USA) included antigen retrieval for 30 minutes and a dilution of 1:500. Omission of the primary antibody served as the negative control and a cervical intraepithelial neoplasm known to express p53 was the positive control. The testing was done using the automated Leica Bond Max (Buffalo Grove, IL, USA) instrument.

Statistical analysis: Student *t*-test, Mann-Whitney U, Chi square and Fisher exact analyses were used, as appropriate, to evaluate for differences between HPV positive and negative tumors and to evaluate factors which associated with a non-favorable response to interferon. *P* values < 0.05 were considered significant.

Results

Demographics of the population and HPV status: Patient age ranged from 31 to 87 years (mean 65 years), and males (n = 18) outnumbered females (n = 9). Twenty-two subjects self-identified as white (85%), and 10 as Hispanic (37%). Five had a previous history of OSSN (19%). Of the 27 tumor samples, 21 (78%) were HPV positive. Twenty patients had only one type of HPV genotype identified. This included HPV 16 in 9, HPV 31 in 4, HPV 33 in 1, HPV 35 in 2, HPV 51 in 1, and a novel HPV in 3. One patient underwent testing on an incisional biopsy specimen prior to starting interferon treatment and was found to have HPV 31 and 51 in the specimen. He failed interferon treatment and his excisional biopsy specimen from the same eye was also analyzed and found to contain HPV 16. Analyzing tumors by HPV status (positive or negative) and by HPV-16 status (positive or negative), no demographic data was associated with the frequency of HPV in a tumor (Table 2).

Clinical features and HPV status: There was a trend for tumors found in the superior limbus to be HPV positive more frequently than tumors in the temporal, nasal, or inferior limbus (Fisher exact *P* = 0.06). (Table 3). HPV 16-

positive tumors, on average, were larger and more likely to have a papillomatous morphology compared to HPV 16-negative tumors (Mann Whitney U $P = 0.08$ and 0.07 , respectively), although again not to a statistically significant level.

p53 and response to interferon by HPV status: Twenty of the 27 specimens underwent concomitant testing for p53 expression (indicative of a mutated protein). No differences in p53 expression were noted by HPV status. Of 26 patients treated with interferon, 24 underwent incisional biopsy prior to treatment and 2 underwent excisional biopsies after failing interferon. Nineteen patients responded to interferon treatment alone and 7 went on to receive other treatments including mitomycin C (MMC), 5-fluorouracil (5-FU), and/or surgical resection. HPV status was not found to correlate with response to interferon treatment (Table 3).

Other metrics and response to interferon: There was a trend for females to have a more favorable response to interferon over men as all females ($n = 9$) responded to therapy while only 10 of 17 males (59%) responded (Fisher exact $P = 0.06$). There was likewise a trend for lesions in the superior bulbar conjunctivae to respond less well to interferon, with 50% of superior lesions ($n = 5$) failing therapy compared to 13% ($n = 2$) of non-superior lesions (Fisher exact $P = 0.07$). None of the other factors examined in Table 3 associated with interferon response with a $p < 0.1$.

Recurrences: Only 1 patient of 27 recurred during our follow up time, which ranged from 0 to 13.6 years after lesion resolution (mean 2.2 years). This

patient was the one who was not treated with interferon during the course of his tumor.

Discussion

Similar to our previous report³, we found a high frequency of HPV in our OSSN tumors with approximately half the genotypes being HPV 16. None of our samples harbored HPV 6 or HPV 11, which is consistent with other studies showing that these genotypes associate with benign ocular lesions.^{28,29} Furthermore, we found that certain clinical features (superior location for all HPV subtypes and a papillomatous morphology for HPV 16) were more common in tumors harboring virus, although with our limited sample size, none of these reached statistical significance. Interestingly, although we hypothesized that HPV positive tumors would have a more favorable response to interferon therapy, we found no difference in response to therapy based on viral status.

HPV associated carcinogenesis has been attributed to its ability to interact with host cellular proteins. Two of its proteins (E6 and E7) can alter the function of critical cellular proteins, such as p53 (enhancing degradation of normal protein).³⁰ In fact, p53 expression (a finding suggestive of a mutated protein) has previously been reported in OSSN.³¹ This is not surprising, however, as sun exposure, another OSSN risk factor,³² is a known inducer of p53 mutations.³³ We found abnormal p53 expression in the majority (70%) of our specimens; however the frequency of p53 expression did not segregate with HPV status. This lack of association is similar to a report by Toth et al. that found no

significant relationship between p53 gene expression (detected in 78% of 23 OSSN) and HPV (detected in 17% of cases).³⁴

HPV has also been shown to affect the epidermal growth factor receptor (EGFR) signaling pathway, a pathway with demonstrated abnormalities in OSSN, and one associated with tumor invasiveness.⁸ Invasive OSSN tumors were more likely to stain positive for proteins involved in the EGFR signaling pathway including cytoplasmic p-MAPK and p-Akt, and nuclear p-EGFR.³⁴ Our study, however, with limited power, did not find a relationship between AJCC clinical grade or pathologic stage and HPV presence. It is also interesting that in one OSSN study, the presence of HPV (detected in 11% of tumors) was associated with significantly improved disease-free survival in 48 OSSN patients all treated with surgical excision.⁹ Unfortunately, with only one local recurrence in our group, we do not have data to answer this question in our patient sample.

As with all studies, our findings need to be considered in light of our study limitations, which included a small sample size with limited follow up and a minimal number of recurrences after treatment. In the future, we hope to further study our findings and the role of HPV in OSSN. Despite these limitations, we found that viral presence was not required for a favorable response to interferon therapy. This is interesting because since interferon has antiviral properties, one might hypothesize that the HPV lesions might in fact be more responsive to IFN.

To the best of our knowledge, this is the first report that attempts to correlate viral presence with response to interferon therapy. The importance of this study is that we found that the presence of HPV had no effect on the

response to treatment with interferon. These findings highlight the need for more research on tumor and patient factors that predict tumor course and response to treatment. These data are essential so as to individualize OSSN treatment and select the therapy that will lead to an optimal clinical outcome, while minimizing patient morbidity, time, and costs.

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339 **Figure legend:**

340 **Figure 1.** Correlation of HPV DNA detection and p53 expression in conjunctival
341 lesions. Panel A shows the positive control for the detection of HPV 16 DNA in a
342 cervical intraepithelial neoplasia grade 1. Note the strong nuclear based signal
343 (arrow). Panel B shows a high grade conjunctival dysplasia also positive for HPV
344 16 DNA by in situ hybridization (arrow). Note that the normal epithelia adjacent
345 to the dysplasia is HPV negative (panel C) which thus serves as an internal
346 negative control. Panel D is a serial section to panel B where the p53
347 immunohistochemistry shows rare positive cells (arrow) typical of productive HPV
348 infection. Panels E and F are serial sections of a different conjunctival high
349 grade dysplasia. Note the intense signal for HPV 31 in panel E whereas the
350 serial section tested for HPV 16 by in situ hybridization shows a weak signal
351 (panel F) reflecting the weak cross homology between HPVs 16 and 31. Panels
352 A-D are at 200X; panels E and F are at 600X.

Figure 1

Table 1: Review of published epidemiology on human papillomavirus (HPV) and ocular surface squamous neoplasia (OSSN)

Author	Location	Population	Technique	HPV frequency
Auw-Haedrich ⁹	Freiburg, Germany	12 OSSN/pts 15 controls	PCR	16.7% CIN (HPV-16) 0% controls
Chauhan ¹⁰	New Delhi, India	64 OSSN/pts 15 controls	PCR	11% OSSN (HPV-16) 0% control
Guthoff ⁵	Wuerzburg, Germany	31 OSSN/pts 11 pterygia 5 controls	IHC, PCR	0% OSSN 0% pterygia 0% controls
Lauer ¹¹	Louisiana, USA	5 OSSN/pts	PCR	80% OSSN (2 HPV-16, 2 HPV-16/18)
Manderwad ¹²	Hyderabad, India	57 OSSN/48 pts	ISH, PCR	0% OSSN
McDonnell ¹³	Los Angeles, USA	48 OSSN/44 pts 6 pterygium	PCR	88% OSSN (HPV-16) 0% controls
Mochizuki ¹⁴	Gifu, Japan	1 OSSN	ISH, PCR	100% OSSN (HPV-16)
Moubayed ¹⁵	Dar es Salaam, Tanzania	14 OSSN	ISH	93% OSSN (12 HPV-6/11/16/18, 1 HPV-18)
Nakamura ⁷	Japan	8 OSSN 9 papillomas	IHC, ISH, PCR	50% OSSN (2 HPV-16, 2 HPV-18) 44% papillomas (HPV-6)
Saegusa ¹⁶	Kitasato, Japan	3 OSSN 16 papillomas	ISH, PCR	38% OSSN (HPV-16) 75% papillomas (HPV-16)
Scott ³	Miami, USA	10 OSSN 5 controls	ISH, PCR	100% OSSN (5 HPV-16, 5 HPV-18) 0% controls
Sen ¹⁷	New Delhi, India	30 OSSN 35 papillomas 30 controls	IHC	0% OSSN 17% papillomas 0% controls
Simbiri ¹⁸	Gaborone, Botswana	28 OSSN 8 pterygia	IHC, ISH, PCR	75% OSSN (6 HPV-6, 13 HPV-11, 17 HPV-16, 15 HPV-18, 7 HPV 31, 1 HPV-33) 75% pterygia (5 HPV 11, 6 HPV 16, 5 HPV 18)
Tuppurainen ¹⁹	Kuopio, Finland	4 OSSN	ISH, PCR	0% OSSN

Woods ²⁰	Sydney, Australia	46 OSSN 42 pterygia 69 controls	IHC, PCR, sequencing	7% OSSN (HPV-16) 0% pterygia 0% controls
Yu ⁸	Uganda and Kenya	38 OSSN	PCR	61% OSSN (17 HPV-18, 6 HPV-16/18)

Pts=patients; PCR=polymerase chain reaction; IHC=immunohistochemistry; ISH=in situ hybridization

Table 2: Demographic information in patients with ocular surface squamous neoplasia (OSSN) by human papillomavirus (HPV) status

Number of eyes/patients	HPV positive	HPV negative	HPV 16 positive	HPV 16 negative	<i>P</i> value/ <i>P</i> value
Age (years), mean [SD]	64 (16)	70 (11)	68 (6.4)	64 (18)	0.45/0.44
Gender, female % (n)	29% (6)	50% (3)	20% (2)	41% (7)	0.37/0.41
Race, white % (n)	80% (16)	100% (6)	78% (7)	88% (15)	0.54/0.59
Ethnicity, Hispanic % (n)	29% (6)	67% (4)	20% (2)	47% (8)	0.15/0.23
Involved eye, right % (n)	33% (7)	50% (3)	30% (93)	41% (7)	0.64/0.69
History of OSSN % (n)	19% (4)	17% (1)	20% (2)	18% (3)	1.0/1.0

n = number of individuals in group; SD = standard deviation

Table 3: Clinical information in patients with ocular surface squamous neoplasia (OSSN) by human papillomavirus (HPV) status

	HPV positive	HPV negative	HPV 16 positive	HPV 16 negative	p-value/ p-value
OSSN Area mm ²	53 (47)	25 (22)	68 (58)	34 (28)	0.18/0.08
AJCC Classification					
T1 % (n)	10% (2)	17% (1)	0% (0)	18% (3)	0.32/0.35
T2 % (n)	19% (4)	0% (0)	20% (2)	12% (2)	
T3 % (n)	71% (15)	83% (5)	80% (8)	71% (12)	
Location*					
Temporal % (n)	52% (11)	33% (2)	50% (5)	47% (8)	0.65/0.88
Inferior % (n)	43% (9)	17% (1)	60% (6)	24% (4)	0.36/0.10
Superior % (n)	48% (10)	0% (0)	50% (5)	29% (5)	0.06/0.29
Nasal % (n)	38% (8)	50% (3)	50% (5)	35% (6)	0.66/0.45
Cornea % (n)	85% (17)	100% (6)	90% (9)	89% (14)	1.0/1.0
Multifocal % (n)	10% (2)	0% (0)	20% (2)	0% (0)	1.0/0.13
Appearance					
Papillomatous % (n)	29% (6)	17% (1)	50% (5)	12% (2)	1.0/0.07
Nodular % (n)	38% (8)	50% (3)	40% (4)	41% (7)	0.66/1.0
Leukoplakia % (n)	43% (9)	67% (4)	50% (5)	47% (8)	0.39/0.88
Gelatinous % (n)	33% (7)	33% (2)	40% (4)	29% (5)	1.0/0.68
Pathological grade					
CIN 1 % (n)	10%(2)	17% (1)	0% (0)	18% (3)	0.84/0.21
CIN 2 % (n)	76 % (16)	67% (4)	80% (8)	71% (12)	
CIN 3 % (n)	14% (3)	17% (1)	20% (2)	12% (2)	
Interferon failure % (n)	30% (6)	17% (1)	33% (3)	24% (4)	1.0/0.66
P53+% (n)	57% (10)	80% (4)	56% (5)	82% (9)	1.0/0.34

*Tumors could involve more than one quadrant, e.g. a tumor that involved the temporal and superior bulbar conjunctivae would be represented both in the temporal and the superior location category.

CIN=conjunctival intraepithelial neoplasia; CIS= carcinoma in situ; SCC=squamous cell carcinoma; AJCC= American Joint Committee on Cancer