The Prevalence, Anatomic Distribution and Significance of HPV Genotypes in Head and Neck Squamous Papillomas as Detected by Real-Time PCR and Sanger Sequencing

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Abstract

Squamous papillomas (SPs) of the head and neck are generally regarded as a human papillomavirus (HPV)-driven process, but reported rates of HPV detection vary dramatically. Moreover, they are generally considered a benign condition, but the detection of high risk HPV types is commonly reported. This latter finding is particularly disturbing to clinicians and their patients given the alarming rise of HPV-associated head and neck cancer. The capriciousness of HPV detection reflects in large part differences in methodologies. The purpose of this study was to review an institutional experience using a state of the art detection method to determine the presence, type and anatomic distribution of HPV in head and neck SPs. The surgical pathology files of the Mount Sinai Hospital were reviewed for all SPs that had undergone HPV testing between 2012 and 2018. HPV screening was performed on tissue blocks with real-time PCR using primers designed to target the L1 region of low and high-risk HPV types. Genotyping was performed on HPV positive cases. HPV detection was repeated for cases that were originally reported to be positive for high risk HPV. 134 cases had undergone HPV analysis. Of the 131 with sufficient cellular material, 2 were excluded because the HPV testing yielded inconclusive results. The remaining 129 cases were the basis of this study. Thirty-eight cases (29%) were HPV positive and 91 (71%) were negative. The most common genotype was HPV 6 ($n = 27$, 71%), followed by HPV 11 ($n = 10$, 26%). One case (1%) was HPV positive but the genotype could not be determined. Of the HPV negative cases, 3 were originally reported as HPV 16 positive but found to be HPV negative on re-review and repeat testing. SPs arising in the larynx were more likely to harbor HPV than those arising in the oral cavity and oropharynx (64% vs. 10%, $p < 0.00001$). Similarly, recurrent respiratory papillomatosis (RRP) were much more likely to be HPV positive than solitary SPs (71% vs. 10%, $p < 0.00001$). Almost a third of head and neck SPs harbor HPV, but incidence is highly dependent on anatomic site. Those arising in the larynx are more prone to be HPV-driven than those arising in the oral cavity and oropharynx, particularly when occurring in the setting of RRP. High risk HPV could not be confirmed in any of the cases. Routine HPV testing as a strategy to unmask potentially malignant lesions harboring high risk HPV is not likely to be useful.

Keywords Human papillomavirus · p16 · Recurrent respiratory papillomatosis · Squamous cell papilloma

Introduction

Over 200 human papillomaviruses have been identified to date. In the head and neck, most clinically relevant types belong to the alpha-papillomavirus genera capable of

infecting mucosal and cutaneous sites. The genus is subdivided into low risk-cutaneous, low-risk mucosal and high-risk mucosal types based on their tropism and ability to cause cancer [1]. Low risk types have been implicated in the etiology of benign mucosal lesions of the head and neck such as squamous papilloma (SP), sinonasal exophytic papillomas, verruca vulgaris, multifocal epithelial hyperplasia and laryngeal papillomatosis. High risk types, particularly type 16, have been associated with a form of head and neck cancer involving the oropharynx. In the United States, the incidence of these HPV-positive oropharyngeal squamous cell carcinomas continues to rise, with a threefold increase

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from 1988 to 2004 [2] and surpassing the cervix as the most common site of HPV-related cancer [3].

The characterization of an HPV-related oropharyngeal carcinoma as an important and distinct form of head and neck cancer can be largely attributed to the refinement of HPV detection strategies that have permitted accurate detection of HPV and the reliable distinction between causal and non-causal (e.g. passenger virus and viral contaminant) forms [4]. The precise distribution of low risk HPV in head and neck SPs could likewise benefit from state of the art detection assays. Vast variation in the reporting of HPV status has resulted in widely disparate views of the role of HPV as a pathogenic agent in SP. On the one hand, HPV has been dismissed as an etiologic factor of SPs, largely based on similar detection rates in normal oral mucosa and lesional tissue [5]. On the other hand, HPV has been implicated as an etiologic driver of carcinomatous transformation. This later concern has been incited by three trends: (1) The alarming rise in HPV-related cancers of the oropharynx designated by some as an “epidemic of virus-induced carcinoma” [6]; (2) the frequent finding of high risk types of HPV in SPs [7–11]; and (3), the growing importance of HPV detection and genotyping at other mucosal sites such as the cervix [12] and anus [13, 14] as a way to predict carcinomatous transformation. The purpose of this study was to clarify the frequency and distribution of HPV in SPs of the head and neck, determine the precise HPV genotype for positive cases, and better inform the clinical practice of HPV testing as a means of assessing cancer risk for patients with histologically benign SPs.

Patients and Methods

Patients

Study approval was obtained from the Mount Sinai Hospital Institutions Internal Review Board. All head and neck squamous cell papillomas (SPs) that had undergone HPV testing at the Mount Sinai Hospital between 2012 and 2018 were identified through a search of the pathology files. Pertinent clinical, demographic and pathologic information including p16 immunohistochemical staining results and anatomic site was obtained from the pathology database and the electronic medical records. Recurrent respiratory papillomatosis (RRP) was defined as multiple confluent SPs carpeting a contiguous area of the mucosa and/or recurrence of SPs at the same anatomic site over time.

For those cases that were positive for a high risk HPV genotype, (1) the slides were reviewed in an effort to identify any morphologic features of premalignant (i.e. dysplastic) alterations, (2) p16 immunohistochemistry was performed because strong and diffuse staining is accepted as a surrogate

marker for the presence of high risk HPV [15], (3) PCR results of the initial testing were reviewed, and (4) PCR testing was repeated on freshly cut sections from the archival tissue blocks.

P16 Immunohistochemistry

SPs that were initially identified as high risk HPV positive were selected for p16 immunohistochemistry in an effort to confirm the presence of active HPV. Tumor blocks were cut at five-micron thickness, deparaffinized, and subjected to antigen retrieval using 10 mM citrate buffer at 92 °C for 30 min. Immunohistochemistry for p16 was performed using a mouse monoclonal antibody (MTM Laboratories, Heidelberg, Germany) and staining was visualized using the Ultra view polymer detection kit (Ventana Medical Systems, Tucson, AZ) on a Ventana BenchMark XT autostainer (Ventana). Staining was performed according to manufacturer’s instructions in the presence of appropriate controls. Per established diagnostic protocols, only those cases that demonstrated diffuse staining (strong nuclear and cytoplasmic positivity in > 75% of tumor cells) were reported as positive for p16 overexpression [15].

Human Papillomavirus DNA Extraction and Quantification

The Maxwell 16 formalin-fixed paraffin-embedded Tissue LEV DNA Kit (Promega, Madison, WI) was used for DNA extraction from formalin-fixed paraffin-embedded tissue sections, in accord with the manufacturer’s instructions. The concentrations and quality of extracted DNAs were measured by a NanoDrop ND-2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Human Papillomavirus DNA Genotyping

HPV genotyping was performed in a stepwise fashion. An initial HPV screening is performed by consensus PCR by amplifying the HPV L1 region using consensus primers GP5+ and GP6+ and the LightCycler 480 High Resolution Melting Master kit. The L1 region is conserved in over 31 different genotypes including low-risk and high-risk HPV types found in cervical and head and neck cancer (HPV 6, 11, 13, 16, 18, 26, 30, 31, 32, 33, 34, 35, 39, 40, 42, 45, 51, 52, 53, 56, 58, 61, 62, 66, 67, 68, 69, 74, 81, 90, 91) [16, 17]. If HPV DNA is amplified in the sample, a High Resolution Melting Curve Analysis (HRMCA) is performed on the LightCycler 480 instrument to confirm the presence of HPV. Positive samples are reflexed for HPV16/18 genotyping using HPV 16 and HPV 18 specific PCR. Beta-actin was amplified simultaneously as a housekeeper gene to evaluate the DNA integrity and quantity.

For type specific detection of HPV, real-time PCR by LightCycler 480 instrument is utilized to amplify E6 regions of HPV16 and 18. Amplicons are detected by two specific pairs of fluorescence hybridization probes, consisting of four different short oligonucleotides that hybridize to an internal sequence of the amplified fragments of HPV16 and 18 during the annealing phase of the amplification cycle. Two probes are labeled at the 5'-end with LightCycler-Red 640 for HPV16 or LightCycler-Red 670 for HPV18, and to avoid extension, modified at the 3'-end by phosphorylation. The other probes are labeled at the 3'-end with fluorescein. After specific hybridization to the template DNA, two probes of each target come in close proximity, resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores. The emitted fluorescence of LC-Red 640 or LC-Red 670 is then measured by the LC480 Instrument.

In the event HPV16/18-specific PCR was negative, Sanger Sequencing was performed to detect other HPV genotypes after amplification with GP51 and GP61 primer pairs. HPV subtypes other than HPV16/18 were determined by aligning sequences in GenBank.

Results

One hundred thirty-four squamous papillomas (SPs) had undergone real time PCR analysis for the presence of HPV. Of the 131 with sufficient cellular material, 2 were excluded because the HPV testing yielded inconclusive results. The remaining 129 cases were the basis of this study (Table 1). Thirty-eight cases (29%) were HPV positive (Fig. 1) and 91 (71%) were negative. The most common genotype was HPV 6 ($n=27$, 71%), followed by HPV 11 ($n=10$, 26%) (Fig. 1). One case (1%) was HPV positive but the genotype could not be determined. The HPV 6 positive cases were from 20 males and 7 females with ages ranging from 5 to 69 years. Twenty-four occurred in the larynx, 2 in the oropharynx and 1 in oral cavity. The HPV 11 positive cases were from 9 males and 1 female; and their ages ranged from 28–68 years. Six occurred in the larynx, 3 in oral cavity and 1 in the oropharynx. SPs arising in the larynx were more likely to harbor HPV than those arising in the oral cavity and oropharynx (64% vs. 10%, $p<0.00001$, Fischer exact), but the HPV genotypes did not significantly vary across anatomic subsites ($p=0.2$). There were more males ($n=94$, 73%) than females ($n=35$, 27%), but SPs from males were not statistically more likely to harbor

Table 1 Correlation of HPV status of squamous papillomas with demographic and clinical features

	HPV status					
	Negative (n=91, 71%)		Positive (n=38, 29%)			
			High risk (n=0)	Low risk type (n=38)		Overall (n=38)
				6 (n=27, 71%)	11 (n=10, 26%) Unknown (n=1, 3%)	
Sex (n=129)						
Males	64 (68%)	0	20	9	1	30 (32%)
Females	27 (77%)	0	7	1	0	8 (23%)
Site (n=129)						
Oral cavity	15 (79%)	0	1	3	0	4 (21%)
Oropharynx	59 (94%)	0	2	1	1	4 (6%)
Larynx	17 (36%)	0	24	6	0	30 (64%)
Clinical classification (n=92)						
RRP (n=41)	12 (29%)	0	23	6	0	29 (71%)
Oral cavity	0	0	0	0	0	
Oropharynx	0	0	0	0	0	
Larynx	12	0	23	6	0	
Solitary SP (n=51)	46 (90%)	0	1	3	1	5 (10%)
Oral cavity	4	0	0	2	0	
Oropharynx	40	0	1	1	1	
Larynx	2	0	0	0	0	

HPV human papillomavirus; RRP recurrent respiratory papillomatosis; SP squamous papilloma

Fig. 1 This squamous papilloma of the larynx (inset, hematoxylin and eosin stain) was found to be HPV positive based on PCR amplification of the consensus HPV L1 region within the amplification cycle threshold. Also shown are the amplification curves for the HPV 16 and HPV 18 controls. By Sanger's sequencing (not shown), HPV 11 was detected in the SCP

HPV than SPs from females. P16 immunohistochemistry was reported for 26 of the SPs, and all were negative.

Of the HPV negative cases, 3 were originally reported as HPV 16 positive. Microscopic review showed conventional SCP without any dysplastic features. P16 immunohistochemistry showed some weak patchy positivity, but the cases lacked the strong and diffuse positivity that has been associated with high risk HPV infection. Review of the original PCR amplification curves showed no signal amplification in one case. The other two cases showed signal amplification, but only at the outer limits of signal cycle threshold (40 amplification cycles) suggesting a viral contaminant. Repeat PCR testing of these two cases from freshly cut tissue sections did not detect HPV (Fig. 2). Based on these findings, the original test results were regarded as false positives. In effect, high risk HPV was not detected in any of the SPs.

The lesions could be reliably separated into solitary SPs and RRP in 92 cases based on clear documentation in the medical records. Of these 92 cases, 41 were RRPs and 51 were solitary SPs. Twenty-nine of the RRP cases harbored HPV (HPV 6=23, HPV 11=6), but only 5 of the solitary SPs harbored HPV (HPV 6=1, HPV 11=3, HPV undetermined=1) (71% vs. 10%, $p < 0.00001$, Fischer exact). Of note, only two laryngeal lesions were unassociated with RRP, and both were HPV negative.

Discussion

The reported incidence of HPV in SPs of the head and neck has varied widely. In the oral cavity, for example, HPV detection rates have ranged from 0–100%. This variation may be due to some large degree by differences in HPV detection. Keeping in mind that the prevalence of oral HPV among healthy individuals without SPs is approximately 5% [18, 19], we used a highly sensitive real time PCR approach designed to reject incidental background HPV infections not meeting amplification threshold criteria for clinically relevant HPV-induced disease. We found an overall HPV prevalence of 29%. The absence of HPV in the majority of SPs points to the presence of some non-viral mechanism inducing these lesions, or the presence of other HPV types beyond the range of our detection assay. Our detection assay was geared toward the detection of the mucosal tropic virus falling in the alpha genera of HPV. The development of new HPV PCR detection assays, however, have recently disclosed entirely novel HPV types in the oral cavity [20]. Others have shown that some of the cutaneous HPV genotypes among the beta and gamma groups can also exhibit tropism for the oral cavity [10, 21]. The significance of these beta and gamma genotypes

Fig. 2 This HPV negative squamous papilloma lacked dysplastic features (**a** hematoxylin–eosin stain) and was p16 negative (**b** p16 immunohistochemical stain). It was originally recorded as HPV 16 positive based on PCR amplification at the outer limits of amplification cycle threshold (**c** real-time PCR for HPV 16 and HPV 18 positive con-

trols and squamous cell papilloma sample). Repeat PCR testing from freshly cut tissue sections did not detect HPV (**d** real-time PCR for HPV 16 and HPV 18 positive controls and squamous cell papilloma sample)

in the oral cavity has only been weakly investigated. Our findings highlighting a large percentage of SPs without any evidence of an alpha HPV etiology heightens the call for further work on cutaneous HPV in mucosal lesions of the head and neck.

Most studies that have investigated the prevalence of HPV in SPs have been site specific, focusing on the oral cavity, oropharynx or larynx. By including all of these sites, we were able to show that HPV is not uniformly distributed among head and neck SPs. SPs arising in the larynx were much more likely to harbor HPV than those arising in the oral cavity and oropharynx (64% vs. 10%, $p < 0.00001$). This variation likely reflects some combined effects of transmission modes and tissue susceptibility. The glottis portion of the larynx, for example, harbors a squamo-columnar

junction similar to the transformational zone targeted by HPV in the female cervix.

Although the larynx was found to be a relative hot spot for HPV driven SPs, HPV was not detected in over one-third of these laryngeal SPs. Even for those patients with well documented recurrent respiratory papillomatosis (RRP), a process known to be driven by low risk HPV, 29% were HPV negative. This finding is of some concern as current HPV preventive and therapeutic vaccines with their restricted coverage of certain low and high risk alpha genotypes will have limited value in preventing the morbidity associated with SPs including RRP [22, 23]. Current HPV vaccines may be very effective in protecting young previously uninfected women against incident HPV-6 and HPV-11 infections, but these same immunized women may not be fully protected

against having a child developing a non-HPV 6 or 11 form of laryngeal SP [24].

The distribution of HPV types did not significantly vary across anatomic subsites of the head and neck. Consistent with other studies, HPV 6 was more common than HPV 11 in both the larynx and non-laryngeal sites [25, 26]. This predominance of HPV 6 over HPV 11 mirrors its distribution in genital warts and cervical intraepithelial neoplasia and supports the view that the anogenital tract may represent an important reservoir for the spread of HPV to the head and neck [27, 28].

Not only do non-quantitative PCR assays tend to overestimate the incidence of HPV infections, but they may also overrate the contribution of high risk HPV genotypes. In pooled results from 10 published studies identifying high risk HPV in SPs using conventional PCR-based assays, high risk HPV was detected in 19% of HPV positive SPs (63 of 339 cases) for an overall incidence of 11% (63 of 560 total cases) [5, 7, 10, 11, 26, 29–33]. These findings seem to indicate that a significant subset of SPs harbor carcinogenic types of HPV—a sobering observation given the soaring incidence of HPV-driven squamous cell carcinoma of the head and neck cancer. Indeed, this association may be driving the growing clinical practice of testing benign SPs for the presence of high risk HPV along the same lines of using HPV testing as a screening tool for identifying patients at risk of developing cancers of the cervix and anus [12–14]. But just like low risk HPV, high risk HPV can also be detected in oral rinses from normal individuals. In the vast majority of these individuals the HPV infection is cleared without causing cancer [18]. Using a real-time PCR approach to help distinguish clinically relevant from extraneous HPV infections, we did not identify high risk HPV in any of our 129 SPs. Of the three cases initially reported as positive for high risk HPV, rigorous re-evaluation and even repeat testing showed that these results were in fact false positive, likely due to a viral contaminant. Our results are consistent with a recent European study that utilizing real-time PCR in combination with P16(INK4A) immunohistochemistry to draw the same conclusion that oral and oropharyngeal papillomas are not associated with high-risk human papillomavirus infection [34]. Our study was not designed to follow patients over extended periods of time, but the consistent absence of high risk HPV in a large number of SPs suggests that routine testing of benign SPs would not be effective as a screening tool to predict malignant transformation. Omland et al. [26] did report extended follow-up in their patients with HPV-typed laryngeal papillomatosis. They found that neither low nor high risk HPV was helpful in identifying those patients who would go on to develop laryngeal cancers.

In summary, low risk HPV accounts for some SPs of the head and neck, but a sizeable proportion are negative

for HPV 6 and 11. Most HPV positive SPs localize to the larynx in patients with laryngeal RRP, and are consistently with low risk HPV types. Indeed, high risk HPV is conspicuously absent in SPs. The clinical practice of routine screening for high risk HPV in SPs should be discouraged as HPV-induced malignant transformation is not a common pathway accounting for the rising incidence of HPV-related squamous cell carcinoma of the head and neck.

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