Use of In Situ Hybridization for HPV in Head and Neck Tumors: Experience from a National Reference Laboratory

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Abstract The human papillomavirus (HPV) status of head and neck squamous cell carcinomas (SCCs) is a frequent request for Anatomic Pathology labs. However, prognostic value of HPV status is limited to identification of high risk HPV in oropharyngeal SCCs. The purpose of this study is to investigate the ordering practices of in situ hybridization (ISH) for HPV in head and neck tissues at our national reference laboratory. All testing orders for low risk, high risk, and combined low and high risk HPV–ISH tests requested at ARUP Laboratories between January 2010 and November 2013 had their results reviewed and were grouped by anatomic location of the tested tissue. The H&E and HPV–ISH slides from a sample of the most recent 123 tests were reviewed by two pathologists. A total of 1,128 HPV–ISH tests were ordered during the study period. Testing for combined low and high risk HPV was the most commonly ordered test. The positivity rate for high risk HPV was highest in oropharyngeal tissues. 49 of 123 reviewed cases had testing requested on non-malignant tissue, 11 of which were non-neoplastic. Unnecessary HPV–ISH ordering is prevalent in head and neck tissues. Dual testing for low and high risk HPV, frequent testing outside of the oropharynx, and testing non-neoplastic tissues appear to be common practices.

Keywords HPV · In situ hybridization · Ordering · Head and neck

Introduction

Traditionally, squamous cell carcinoma (SCC) of the head and neck primarily affects older individuals with a history of smoking and alcohol use and does not respond favorably to chemoradiation therapy [1]. Multiple studies have proved human papillomavirus (HPV) infection to be an independent risk factor for head and neck SCC [2]. In addition to being a risk factor for a subset of these carcinomas, the presence of HPV defines a distinct subgroup with a better response to treatment and a favorable outcome independent of lymph node involvement, stage, age, or treatment strategy [3]. In recent years, HPV-related head and neck SCC have been dramatically increasing, affecting middle to older aged men with no history of smoking or drinking. HPV positive SCC represents a subset of head and neck tumors classically showing non-keratinizing squamous cell morphology [4]; however, a variety of other morphologies (papillary, adenosquamous, lymphoepithelial-like) have also been described in HPV-related carcinomas [5–8]. HPV-related head and neck SCC are associated with high risk HPVs (most commonly type 16) and even in the setting of a clinically undetectable primary may present with large metastatic lymph nodes in the neck. Low risk HPV types, most commonly types 6 and 11, are associated with laryngeal papillomatosis but do not lead to cancer.

There are currently a variety of HPV detection systems including PCR and in situ hybridization (ISH). Approximately 35 % of all head and neck tumors have been reported to be HPV positive by PCR. In the oropharynx the rate reported is as high as 70–80 % in some studies from

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the US [9, 10]. While DNA-based PCR methods are highly sensitive, they may not be able to distinguish biologically irrelevant HPV (e.g., viral contamination) from clinically significant (or integrated) HPV [11]. HPV DNA ISH testing, on the other hand, correlates with biologically and transcriptionally active HPV and is being utilized as one of the primary modalities for the evaluation of HPV status in head and neck tumors [12].

Additionally, immunohistochemical testing for p16 (which is upregulated in HPV-infected cells) is considered a useful surrogate marker for HPV positivity [12, 13]. Testing for HPV via DNA ISH along with p16 immunohistochemistry is widely used following initial diagnosis of head and neck SCC for prognostic purposes. The purpose of this study is to review utilization of HPV–ISH in head and neck tissues in our national reference laboratory.

Materials and Methods

HPV–ISH requests sent to our laboratory between January 2010 and November 2013 were identified. All of the HPV–ISH requests during this study period submitted to ARUP Laboratories were identified. Subsequently, tests ordered on the head and neck sites were selected. Our laboratory offered the INFORM HPV III Family 16 Probe for high risk HPV (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66) and the INFORM HPV II Family 6 probe for low risk HPV (HPV 6 and 11) (Ventana, Tucson, AZ) during the study period. Clients had the option of requesting low risk (LR), high risk (HR), or the panel (high risk + low risk). The test was interpreted following the manufacturer's guidelines. The determination of a true positive required either the episomal (large, homogeneous, globular navy blue precipitate with the cell nuclei) or integrative pattern (discrete, stippled navy blue nuclear pattern) in the area of interest. The determination of a true negative result required finding no signal within lesional epithelial cells. Appropriately reacting controls were also required for a definitive interpretation to be rendered.

Information on the biopsy site, patient age and gender were provided by the referring pathologists. The type of HPV–ISH test ordered, the biopsy site, and the HPV result were recorded for each test. The biopsy sites were divided into groups by location: larynx, hypopharynx, oropharynx + base of tongue + tonsil, oral cavity, nasopharynx, sinonasal, and neck mass. Since the number of HPV tests requested in head and neck lesions during the study period was high ($n = 1,128$), a small sample (the most recent 125 consecutive cases) was selected for review of H&E and HPV–ISH slides by two pathologists (BLW, HEG). In our H&E review, cases of squamous cell carcinoma were classified as either keratinizing or nonkeratinizing type. To

Table 1 Number of requested HPV–ISH tests in head and neck

	Panel (HR + LR) (%)	High risk (HR) (%)	Low risk (LR) (%)	Total (%)
Larynx	196 (60)	123 (38)	6 (2)	325 (100)
Hypopharynx	4 (67)	2 (33)	0 (0)	6 (100)
Oropharynx, BOT, tonsil	217 (63)	127 (36)	1 (1)	345 (100)
Oral cavity	175 (57)	129 (42)	2 (1)	306 (100)
Nasopharynx	4 (67)	2 (33)	0 (0)	6 (100)
Sinonasal	14 (67)	6 (29)	1 (4)	21 (100)
Neck mass	57 (48)	62 (52)	0 (0)	119 (100)

BOT base of tongue

achieve this the 2005 WHO criteria describing keratinizing squamous cell carcinoma were used [14]. The WHO states that in keratinizing squamous cell carcinoma, most of the tumor shows obvious squamous differentiation in the form of intercellular bridges and/or keratinization (eosinophilic glassy cytoplasm), which includes but does not require keratin pearl formation. Nonkeratinizing squamous cell carcinomas (NKSCC) were defined as carcinomas wherein the above features were not present in the majority of the tumor. 2 of 125 cases were from skin (of the head and neck region) and were excluded from analyses.

Results

Our laboratory processed 2,506 HPV–ISH tests (including low risk, high risk and combined/panel) from private labs/hospital centers, University Hospitals, Children's Hospitals, and VA Medical Centers nationwide between January 2010 and November 2013. Our low risk testing was the least frequently ordered with a total of 85 tests from 46 different clients. The mean number of tests per client was 1.8 (range 1–8). Our high risk testing included a total of 942 tests from 170 different clients. The mean number of tests per client was 5.5 (range 1–252). Our combined high and low risk (Panel) HPV testing was the most frequently ordered HPV test with a total of 1,449 tests from 175 clients. The mean number of tests per client was 8.1 (range 1–134). Of the HPV–ISH tests, 1,128 were ordered on head and neck lesions, which included 667 panel (high + low risk), 451 high risk (HR), and 10 low risk (LR) HPV tests. The number and type of HPV–ISH tests requested by location are summarized in Table 1. Overall, the panel HPV–ISH was the most common test ordered in head and neck lesions (667/1,128; 59.1 %). With the exception of neck masses where high risk HPV–ISH was ordered the most, the panel test was the most frequently ordered HPV test per anatomic site (Table 1). The high risk HPV–ISH

Table 2 HPV–ISH positivity by site for high and low risk HPV

	Positive/total high risk test (%)	Positive/total low risk test (%)
Larynx	22/319 (6.9)	119/206 (57.8)
Hypopharynx	2/12 (16.7)	0/8 (0)
Oropharynx, BOT, tonsil	180/344 (52.3)	0/56 (0)
Oral cavity	67/294 (22.8)	12/167 (7.2)
Nasopharynx	2/8 (25)	0/4 (0)
Sinonasal	4/20 (20)	2/15 (13.3)
Neck mass	39/109 (35.8)	1/57 (1.8)

BOT base of tongue

Table 3 Types of HPV–ISH tests requested in head and neck lesions by histologic diagnosis

Diagnosis	Positive/HR HPV tested (%)	Positive/LR HPV tested (%)
Malignant (n = 74)		
KSCC (n = 55)	26/55 (47.3)	0/21 (0.0)
NKSCC (n = 14)	9/14 (64.3)	2/11 (18.2)
PSCC (n = 3)	3/3 (1.0)	0/1 (0.0)
Undifferentiated CA (n = 1)	0/1 (0.0)	0/1 (0.0)
SCCIS (n = 1)	0/1 (0.0)	0/0 (0.0)
Total	38/74 (51.4)	2/34 (5.9)
Benign (n = 49)		
Squamous Papilloma (n = 37)	2/37 (5.4)	16/34 (47.1)
Squamous Hyperplasia (n = 6)	1/6 (16.7)	0/4 (0.0)
Normal Epithelium (n = 3)	0/3 (0.0)	0/3 (0.0)
Laryngeal Nodule (n = 2)	0/2 (0.0)	0/2 (0.0)
Sinonasal Papilloma (n = 1)	0/1 (0.0)	0/1 (0.0)
Total	3/49 (6.1)	16/44 (36.4)

KSCC Keratinizing squamous cell carcinoma, *NKSCC* nonkeratinizing squamous cell carcinoma, *PSCC* papillary squamous cell carcinoma, *SCCIS* squamous cell carcinoma in situ

positivity rate was highest in oropharynx + base of the tongue (BOT) + tonsil group, and low risk HPV–ISH positivity rate was highest in larynx (Table 2).

In our review of the H&E and HPV–ISH slides from a small sample group of the most recent cases, we had 100 % concordance (among two pathologists) in malignant versus benign, and HPV–ISH positive versus negative categories. 28/74 (37.8 %) of malignant head and neck lesions reviewed were from BOT + oropharynx + tonsil. 12/28 (42.8 %) of carcinomas from BOT + oropharynx + tonsil were of NKSCC type and 2/28 (7.1 %) were of papillary squamous cell types.

Twenty-six out of 55 (47.3 %) cases of keratinizing SCC had HR-HPV by ISH. It should be noted that our concordance between keratinizing and nonkeratinizing morphology was 84 % between two pathologists. All of the patients in this sample group (123/123, 100 %) had high risk HPV testing ordered and 78/123 (63.4 %) had low risk HPV testing ordered irrespective of location or diagnosis (malignant vs. benign). During our review, we identified 11 cases of non-neoplastic tissue biopsies (six squamous hyperplasia, three normal squamous epithelium, and two laryngeal nodule) that had HPV testing ordered (Table 3).

Conclusion

Although there is some data suggesting importance of high risk HPV in sinonasal cancers and undifferentiated carcinoma of oropharynx, it is generally accepted that prognostic significance of HPV is confined to SCC of the oropharynx [8, 15, 16]. Testing the HPV status of benign epithelial lesions of the upper respiratory tract and oral cavity, including solitary squamous papillomas, is of limited clinical utility as solitary squamous papillomas are thought to be unrelated to HPV and are usually cured by simple excision. One recent study by McCord et al. [17] showed that there was no relationship between low or high risk HPV positivity and potentially malignant atypical oral papillary lesions. Also in their study, only 1 out of 24 conventional oral squamous papillomas was positive for low risk HPV and none showed positivity for high risk HPV. Furthermore, in instances of laryngeal papillomatosis where it is understood that the lesions are caused by HPV, there seems to be little clinical utility in pursuing HPV testing. In their recent study of 85 patients with laryngeal papillomatosis, Davids et al. found that the majority of patients (62 %) were positive for low risk HPV [6, 11]. While all of the patients with high grade dysplasia were positive for low risk HPV, there was no association between high risk HPV subtypes (16, 18) and predisposition to dysplasia [18].

Our study shows unnecessary HPV–ISH testing is common in head and neck lesions, and ordering a panel that includes both high and low risk HPV groups is the most common cause of over testing. In addition, high risk HPV testing was indiscriminately ordered in locations outside of the oropharynx, as HPV testing in the oropharynx accounted for only 30.6 % (345/1,128) of the total ISH tests ordered. Other causes of misdirected testing were identified upon a review of a small subset of the most recent test requests. Approximately 9 % (11/123) of the reviewed cases sent for HPV–ISH testing demonstrated tissues showing normal epithelium or non-neoplastic lesions (squamous hyperplasia and singer's nodules). These

tests were ordered by a wide range of clients around the nation, and in our study it appears that HPV testing of non-neoplastic lesions is not rare. 30/123 (23.4 %) audited cases were from BOT + oropharynx + tonsils, and neck masses with a malignant diagnosis qualifying them for high risk HPV testing. However, all (123/123) of the cases reviewed had high risk HPV test ordered. 78/123 (63.4 %) of the cases, including 37 squamous papillomas, had low risk HPV test ordered. In this small sample of 123 cases we received within a period of <2 months, a total of 171 (93 HR HPV and 78 LR HPV) unnecessary HPV–ISH tests were ordered with direct cost of \$21,375, excluding professional fees.

HPV status determination is important in terms of prognosis and in the near future will likely direct specific therapies. There is a general agreement that all oropharyngeal SCC should be tested for the presence of high-risk HPV. Although typical HPV-related SCC is described as nonkeratinizing SCC, keratinizing and hybrid carcinomas are also known to be HPV-related and keratinizing SCC should not be excluded from HPV testing on histologic basis alone [4]. It is worth noting that subclassifying squamous cell carcinomas outside of the oropharynx (where a nonkeratinizing morphology suggests an association with HPV) or nasopharynx (where a nonkeratinizing appearance usually coincides with an Epstein-Barr Virus (EBV) etiology) is not performed in standard pathology practice [14, 19]. In this study we performed this assessment on squamous carcinomas at all locations in order to gauge the interobserver agreement of this subclassification, as well as to determine how predictive a nonkeratinizing morphology is for HPV-positivity at all anatomic sites. Our findings further support that high risk HPV testing on keratinizing SCC of the oropharynx should be performed especially since there is significant interobserver variability for this morphologic feature. HPV testing in metastatic neck nodes/masses may also be justified in many instances as it may suggest the primary tumor site as well as play a role in risk stratification.

Finally, our results show significant incidence of HR HPV–ISH positivity in sites outside of oropharynx. 136/762 (17.8 %) of head and neck biopsies outside of the oropharynx were positive for high risk HPV by our assay, with oral cavity biopsies having the highest rate of non-oropharyngeal sites for HR HPV positivity (67/294; 22.8 %). Overall our study had a 28.5 % (316/1,106) rate of HR HPV positivity at all sites (including the oropharynx) with a rate of 52.3 % (180/344) for oropharyngeal sites. Rates of HPV positivity in the literature vary depending on the detection method used and the tissue types tested. Our rate of HR HPV positivity does not appear to differ significantly from a 2012 cancer registry study by Sethi et al. [20] which had a prevalence of 29.4 %

HPV positivity for carcinomas at all head and neck sites and reported a 50.6 % prevalence within carcinomas of the oropharynx. In the same study by Sethi et al. [20] it was confirmed that HPV positivity (in large part HPV16), was associated with improved survival and that the survival advantage of HPV-positivity was limited to oropharyngeal sites. Thus, in the absence of evidence for a prognostic role of HPV testing outside of the oropharynx and metastatic neck lymph nodes, these locations may not need HPV–ISH testing unless histologic and/or clinical features otherwise suggest HPV related cancers [21, 22]. Utility of low risk HPV testing in head and neck tumors, if any, should also be addressed in future guidelines.

Laboratory testing accounts for nearly 10 % of overall health care costs in the US [23]. Our study demonstrates the need of properly informing clinical teams and pathologists in appropriate utilization of HPV–ISH testing for the purpose of cost containment. Our laboratory recently discontinued panel HPV testing (combined low and high risk) in an attempt to avoid unnecessary test requests and reduce overall cost. The College of American Pathologists guidelines on HPV testing in head and neck tumors, pending at the time of submission of this manuscript, will be a valuable resource.

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Conflict of interest There are no disclaimers or conflicts of interest to report.

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