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Human papillomavirus status of head and neck cancer as determined in cytologic specimens using the hybrid-capture 2 assay

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SUMMARY

Objective: A standardized assay to determine the HPV status of head and neck squamous cell carcinoma (HNSCC) specimens has not yet been established, particularly for cytologic samples. The goal of this study was to determine whether the hybrid capture-2 (HC-2) assay, already widely used for the detection of high risk HPV in cervical brushings, is applicable to cytologic specimens obtained from patients with suspected HNSCCs.

Materials and methods: Fine needle aspirates (FNA) of cervical lymph nodes were pre-operatively obtained from patients with suspected HNSCCs and evaluated for the presence of HPV using the HC-2 assay. HPV analysis was performed on the corresponding resected tissue specimens using p16 immunohistochemistry (IHC) and HR-HPV in situ hybridization (ISH). A cost analysis was performed using the Center for Medicare & Medicaid Services.

Results: HPV status of the cervical lymph node metastases was correctly classified using the HC-2 assay in 84% (21/25) of cases. Accuracy was improved to 100% when cytologic evaluation confirmed the presence of cancer cells in the test samples. The estimated cost savings to CMS using the HC-2 assay ranged from $113.74 to $364.63 per patient.

Conclusions: HC-2 is a reliable method for determining the HPV status of HNSCCs. Its application to HNSCCs may reduce costs by helping to localize the primary site during the diagnostic work-up as well as decrease the interval time of determining the HPV status which would be relevant for providing prognostic information to the patient as well as determining eligibility for clinical trials targeting this unique patient population.

Introduction

In the past decade, significant evidence has established high risk (HR) human papillomavirus (HPV) as the causative agent in an increasing proportion of incident cases of oropharyngeal squamous cell carcinoma (OPSCC) [1–6]. HPV infection is responsible for up to 80% of oropharyngeal cancers in the United States, and over 90% are caused by one viral type, HPV type 16 [7]. Determining the HPV status of OPSCC is important for several reasons. HPV status can provide prognostic information [8–10], assist with localization of the primary site to the oropharynx in patients presenting with metastatic squamous cell carcinoma to the neck [11,12], as well as determine patient eligibility for clinical trials tailored to this cancer population. Despite the importance of discerning HPV status, there is currently no standard approach for HPV testing of clinical samples. Instead, methods of HPV testing across laboratories vary considerably reflecting the biases and tendencies of individual investigators [13]. Current methods for HPV detection in head and neck clinical samples include type-specific polymerase chain

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reaction (PCR), real-time PCR (RT-PCR), immunohistochemical detection of surrogate markers such as p16, and HPV deoxyribonucleic acid (DNA) in situ hybridization (ISH). However, the application of these detection methods to cytologic specimens is very limited [14].

The hybrid capture 2 (HC-2) assay is a commercially available microplate analysis approved by the US Food and Drug Administration for the detection of HPV DNA as part of cervical cancer screening [15,16]. It is a liquid-phase hybridization assay that uses a RNA probe mixture for the detection of 13 high risk types of HPV including type 16 [16,17]. We have previously shown that this assay is a highly reliable method of determining HPV status in brushings and aspirates from surgically resected head and neck squamous cell carcinomas (HNSCCs) [18]; but, unrestricted access to tumor cells at the pathology bench may exaggerate its strengths and understate its limitations as a preoperative tool where true cytologic samples are prone to variability in cellularity and viability. The purpose of this feasibility study is to expand the application of the HC-2 assay to cytologic specimens obtained *in vivo* from patients with metastatic HNSCC as well as perform a cost analysis of applying this assay in clinical practice.

Materials and methods

Patient recruitment

Patients who presented with cervical lymphadenopathy which was palpable on a head and neck examination were screened for eligibility in the Department of Otolaryngology – Head and Neck Cancer clinic at the Johns Hopkins Hospital (Baltimore, MD). Since cervical lymph nodes 1 cm or greater in size are more often palpable and easily aspirated in a clinical setting without the use of ultrasound-guided techniques, only patients with palpable lymph nodes $\geqslant$1 cm were included in this study. After appropriate consent was obtained, twenty-five patients who met the study criteria were enrolled. The clinical study was approved by the Institutional Review Board at the Johns Hopkins Hospital.

Fine needle aspiration biopsy

A fine needle aspiration biopsy (FNAB) of metastatic cervical lymph nodes was performed either in the clinic or in the operating room during an examination under anesthesia of the upper aerodigestive tract. The skin was prepped with an alcohol pad, and a 3 cc syringe (Becton-Dickinson, Franklin Lakes, NJ, USA) with a 25 gauge needle was used to inject 0.2 cc of 1% lidocaine with 1:100,000 epinephrine subcutaneously. The palpable lymph node was secured between two fingers and a 25 gauge needle on a 10 cc syringe was passed percutaneously into the lymph node. With the depressor pulled back to exert negative pressure on the syringe, the needle was passed 3–5 times. The aspirate from the needle was placed onto a Vista Vision HistoBond charged slide (VWR, Arlington Heights, IL, USA). The slide was air-dried and subsequently stained with a Diff-Quik stain. The slide was reviewed by a cytopathologist (ZM) to assess overall cellularity of the specimen. A second pass with a fresh needle was then made into the lymph node, and the aspirate placed into 1 mL of transport medium (Digene/Qiagen Corporation, Valencia, CA, USA) and the vial was stored at −20 °C until the HC-2 assay was performed.

CaSki and SiHa cell lines

Two HPV-associated cancer cell lines, CaSki and SiHa, were obtained from American Type Culture Collection (ATCC). The cells underwent digestion with 20 mg/mL of proteinase K (Roche) in the presence of 1% sodium dodecylsulfate (Bio-rad) at 48 °C for 2 days. DNA was then extracted using UltraPure Phenol:Chloroform:Isoamyl Alcohol reagents (Sigma–Aldrich, USA) following the manufacturer's instructions. DNA was then precipitated in 100% ethanol, centrifuged at 4150 rpm for 45 min, washed in 70% ethanol twice, dissolved in LoTE buffer, and stored at −20 °C. Starting at a concentration of 100 ng of total genomic DNA, the DNA from each cell line then was diluted serially 10-fold.

Hybrid capture 2 liquid-phase assay

A modified HC-2 HPV assay (Digene/Qiagen Corporation) was used to test the FNA samples for the presence of HR-HPV DNA. This test qualitatively screens for 13 HR-HPV types (including 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) by in vitro nucleic acid hybridization with signal amplification using chemiluminescence on a microplate. Briefly, the collected samples in the transport medium were denatured to single-stranded DNA by mixing the samples with the denaturation reagent and incubated in a 65 °C water bath for 45 min. Samples were applied to the hybridization microplate, mixed with the HR-HPV specific RNA probe mixture, and incubated at 65 °C for 60 min. The capture step was then performed by applying the samples to microplate wells coated with RNA/DNA hybrid-specific antibodies and shaken at 1100 rpm at 25 °C for 60 min. The hybrid detection phase was then completed by mixing hybrid samples with alkaline phosphatase-conjugated antibodies (Detection reagent I), and signal amplification performed with incubation with Detection Reagent II. Sample scores were based on a ratio of the relative light unit (RLU) per positive control (CO). A RLU/CO score $\geqslant$2.5 was considered positive for the presence of high-risk HPV DNA. All RLU/CO scores <0.85 were considered negative for the presence of HR-HPV DNA. RLU/CO scores in the range of 0.85–2.5 were interpreted as equivocal for HR-HPV. Both positive and negative calibrators were used per the manufacturer's instructions and were run with each plate. In addition to quality control, calibration was performed with each run to ensure that the reagents and furnished calibrator material were functioning properly.

p16 Immunohistochemistry

HPV status on the resected cancers was confirmed within the tumor by overexpression of p16 which is induced by HPV E7 oncoprotein activity. Briefly, five micron sections of formalin fixed paraffin-embedded (FFPE) tissue were deparaffinized and subjected to antigen retrieval using 10 mM of citrate buffer (92 °C for 30 min).

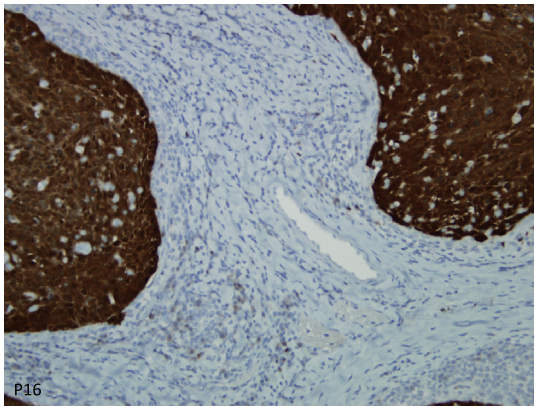

Fig. 1. Human papillomavirus-associated head and neck squamous cell carcinoma confirmed by p16 immunohistochemistry.