Human Papillomavirus-Associated Adenocarcinoma of the Base of Tongue: Potentially Actionable Genetic Changes

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Abstract While human papillomavirus (HPV)-positive squamous and adenosquamous carcinomas of the oropharynx have been well characterized, HPV-associated pure adenocarcinomas are exceptionally rare. Herein we report the clinicopathologic features of one such HPV-associated adenocarcinoma of the base of tongue (BOT). A 70 year-old male presented with a 2.8 cm base of tongue mass and lymphadenopathy. Immunohistochemically, the adenocarcinoma was p63 negative and p16 positive. HPV positivity was shown by in situ hybridization. Features of salivary type tumor or metastasis from a distant primary were absent. IonTorrent™ semiconductor sequencing analysis for 739 cancer-associated mutations in 46 actionable cancer genes was performed and *PIK3CA* exon 9 (p.E545K) and *MET* exon 2 (p.E168D) mutations were identified. No *PIK3CA* or *MET* amplification was identified by fluorescence in situ hybridization. A re-review of archival HPV-positive oropharyngeal squamous cell carcinomas (n = 89, 1983–2013) showed no additional cases of adenocarcinoma. The clinical

follow-up for the three previously reported cases of HPV-associated adenocarcinoma of the BOT was updated. All previously reported cases were tested and were negative for *PIK3CA* exon 9 and 20 and *MET* exon 2 mutations. These findings offer a molecular basis for potential therapeutic use of *PIK3CA* inhibitors in a subset of patients with HPV-associated adenocarcinoma of BOT.

Keywords HPV · p16 · Adenocarcinoma ·
Base of tongue · *PIK3CA* · *MET*

Introduction

Oropharyngeal squamous cell carcinomas (SCC) are increasingly associated with human papillomavirus (HPV) [1, 2]. In addition to the non-keratinizing morphology most commonly seen in oropharyngeal SCC, other variants of SCC, including papillary and lymphoepithelial, have been recognized [3, 4]. Recently, the spectrum of HPV-associated oropharyngeal carcinomas was significantly expanded to include a subset of adenosquamous carcinomas [5],

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neuroendocrine carcinomas [6,7], un-differentiated carcinomas [8] and adenocarcinomas of the base of the tongue (BOT) [9, 10].

There are at least two practical consequences to the above developments. First, in patients with unknown primary, SCCs metastatic to cervical lymph nodes are usually tested for HPV to assess for the possibility of an oropharyngeal source [11]. Our improved understanding of the morphologic spectrum of HPV-associated oropharyngeal carcinomas highlights the need to consider HPV testing in non-squamous cell carcinomas, as well. Second, it suggests that similar to HPV-positive SCC [12, 13], other HPV-associated oropharyngeal tumor types may also have a higher prevalence of phosphatidylinositol 3-kinase (PI3K) pathway abnormalities [14, 15]. This may be especially true for HPV-associated adenocarcinomas of the BOT, since up to 30 % of adenocarcinomas involving the uterine cervix—another site where HPV is found in a variety of carcinomas—were recently shown to harbor mutations in the p110 α catalytic subunit of phosphoinositide 3-kinase (*PIK3CA*) [16].

Herein we present one additional case of HPV-associated adenocarcinoma, not otherwise specified (NOS), of the BOT. The sequencing analysis of this case for 739 hotspot mutations in 46 potentially “druggable” cancer genes revealed *PIK3CA* and *MET* mutations. Since a variety of carcinomas harboring “driver” *PIK3CA* mutations demonstrate enhanced response to PI3K pathway inhibitors [12, 17–19] and since testing for this mutation is routinely available in many clinical laboratories, all previously reported HPV-associated adenocarcinomas, NOS, of the BOT were also tested for *PIK3CA* and *MET* mutations.

Materials and Methods

This study was approved by the Institutional Review Board of the University of Pittsburgh Medical Center (IRB# PRO11010195). HPV-associated oropharyngeal SCC treated from 1983 to 2013 at the University of Pittsburgh Medical Center and identified as part of a prior project (n = 89) [20] were re-reviewed with the knowledge of HPV-associated adenocarcinoma, NOS, of the BOT [9, 10].

Immunohistochemical staining was performed with the antibodies listed in Table 1 according to manufactures' recommendations. For p16 analysis, cases were considered positive if >70 % of tumor cells showed diffuse and strong cytoplasmic and nuclear staining [21].

HPV detection by in situ hybridization was performed using probes targeting 37 distinct HPV subtypes, including 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, and 52 (Y1404; Dako, Carpinteria, CA). High risk HPV detection was performed with Inform HPV III Family 16 Probe (B) (Ventana # 780-4295), Human Papillomavirus DNA Probe Cocktail,

Table 1 Details on antibodies utilized in this study and immunohistochemical results

Antibodies (clone)	Dilution	Vendor	Results
p63 (4A4)	1:200	Thermo	Negative
p16 (INK4alpha)	Prediluted	MTM	Positive
CK5/6 (D5/16 B4)	1:50	Dako	Negative
CK7 (OV TL 12/30)	1:200	Dako	Positive
AE1/3 (AE1/AE3)	1:100	Dako	Positive
Carcinoembryonic antigen, monoclonal (TF 3H8-1)	Prediluted	Dako	Positive
Synaptophysin (NA)	Prediluted	Cell marque	Negative
Thyroid transcription factor-1 (8G7G3/1)	1:50	Dako	Negative
CDX-2 (CDX2-88)	1:200	Biogenex	Negative
Androgen receptor (AR441)	1:100	Dako	Negative
Prostate specific antigen (NA)	Predilute	Ventana	Negative

with an affinity to HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 66. Five-micrometer tissue sections were deparaffinized and digested with proteinase K (Roche Diagnostics, Indianapolis, IN). Cases with punctate nuclear signal were considered positive [21].

The quantity and quality of isolated DNA was adequate for analysis of 739 cancer-associated mutations in 46 actionable cancer genes using IonTorrentTM semiconductor sequencing technology, as previously described [22]. Next generation bidirectional sequencing was performed on the Ion Torrent Personal Genome Machine and analyzed with the Torrent Suite Software v2.2 or v3.2 and NextGENE v2.3.1 (Life technologies, NY, USA). The full list of examined mutations/sequence variants can be found in the supplemental material.

The presence of *PIK3CA* and *MET* mutations was confirmed by Sanger sequencing: DNA was amplified with primers flanking exon 9 of the *PIK3CA* gene (forward primer 5'-TGA AAA TGT ATT TGC TTT TTC TGT-3' and reverse primer 5'-TGT AAA TTC TGC TTT ATT TAT TCC-3') and exon 2 of the *MET* gene (forward primer 5'-AGC GTC AAC AGA GGG ACC TG-3' and reverse 5'-CGA ATG CAA TGG ATG ATC TG-3'). PCR products were sequenced in both sense and antisense directions using the BigDye Terminator v3.1 cycle sequencing kit on ABI 3730 (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions. The sequences were analyzed using Mutation Surveyor software (SoftGenetics, LLC., State College, PA).

PIK3CA Mutation Analysis of the Previously Reported Cases [9, 10]

Tissue cores from tumor targets were obtained as previously described [23] or microdissected from unstained slides under

the guidance of H&E stained slides. DNA was isolated from tissue cores using the DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. *PIK3CA* mutation testing was performed by SNaPshot PCR as per the manufacturer's manual and as previously described [24]. Briefly, primers for exon 9 (forward 5'-AGTAACAG ACTAGCTAGAGA-3' and reverse 5'-ATTTTAGCAC TTACCTGTGAC-3') and exon 20 (forward 5'-GACCCTAG CCTTAGATAAAAC-3' and reverse 5'-GTGGAAGATCCA ATCCATTT-3') were used for amplification. Denatured products were analyzed on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Fluorescence In Situ Hybridization (FISH)

PIK3CA (Spectrum Green) and Chromosomal Enumeration Probe (CEP) 3 (Spectrum Orange) (Abbot Molecular, Des Plaines, IL, USA) FISH was performed as per manufacturer's recommendation. *PIK3CA* amplification was defined as *PIK3CA*/CEP3 >2. FISH for *MET* was performed using a RP11-163C9 probe (Spectrum Orange) from Children's Hospital Oakland Research Institute, Oakland, CA, USA and CEP7 Spectrum Green probe (Abbot Molecular, Des Plaines, IL, USA).

Results

A 70-year-old male, non-smoker, with history of occasional tobacco chewing and social alcohol consumption, presented with a 3 month history of dysphagia and a left neck mass. The computed tomography scan revealed a 2.8 cm mass at the left base of tongue and ipsilateral level II lymphadenopathy (up to 4 cm). This was later confirmed by positron emission tomography scan, demonstrating increased F-18 fluorodeoxyglucose (FDG) uptake at these sites and no evidence of distant metastatic disease. He was staged clinically as cT2N2b and underwent biopsy of the base of the tongue.

The diagnostic biopsy from the left base of the tongue showed an invasive adenocarcinoma involving the submucosa (Fig. 1). The overlying squamous epithelium was unremarkable. The tumor showed cribriform growth, without necrosis. There was no evidence of squamous or neuroendocrine differentiation. The tumor cells were columnar with eosinophilic cytoplasm. Mitoses were readily appreciated. Neither angiolymphatic, nor perineural invasion were identified. The results of immunohistochemical studies confirmed the glandular differentiation and argued against the possibility of a metastasis from a distant occult primary of thyroid, pulmonary, gastrointestinal, prostate or salivary origin (Table 1). Immunostaining for p16 was positive, and in situ hybridization for HPV was positive (punctate pattern), with both probes (Fig. 1).

The patient has undergone concurrent radiation and chemotherapy with weekly carboplatin and paclitaxel. Five months later, imaging studies revealed significant interval decrease in size of the BOT mass with development of ulceration in the tumor bed. A thin rim of soft tissue with moderately increased FDG uptake, likely representing residual viable tumor, was noted. The left level II lymph node has resolved.

Molecular Findings

IonTorrent™ semiconductor sequencing analysis was performed and *PIK3CA* exon 9 (p.E545K, c.1633G > A) and *MET* (p.E168D, c.504G > T) mutations were identified. While *PIK3CA* p.E545K is a well known gain-of-function mutation located in helical (exon 9) domain of the gene at one of the typical hot spots that can be targeted by PI3K/AKT/mammalian target of rapamycin inhibitors, the significance of *MET* p.E168D is uncertain. Other tested mutation hot spots in genes listed below were negative: *ABL1*, *AKT1*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNNB1*, *EGFR*, *ERBB2* and 4, *FBXW7*, *FGFR1-3*, *FLT3*, *GNAS*, *HNFI1A*, *HRAS*, *IDH1*, *JAK2* and 3, *KDR*, *KIT*, *KRAS*, *MLH1*, *MPL*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, *PTEN*, *PTPN11*, *RBI*, *RET*, *SMAD4*, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53*, *VHL* (see supplemental file for details). The coverage of the following gene hot spots was below the laboratory cut-off of 300X, which is of insufficient quality to make an accurate call: *FGFR3* p.R248C, *NOTCH1* p.V1579del and p.L1575P, *RET* p.E768D, *ATM* p.L2890V, *RBI* p.L199*.

The semiconductor sequencing analysis revealed that 44 % of *PIK3CA* alleles were mutant (with the remaining 56 % represented by the wild type allele). Thirty-seven percent of *MET* alleles were mutant. Since mutations in some oncogenes may be accompanied by amplification, *PIK3CA* and *MET* FISH were performed. No amplification of *PIK3CA* (*PIK3CA*/CEP3: 1.4) or of *MET* (*MET*/CEP7: 1.3) was identified.

HPV-Associated Adenocarcinomas, NOS, of the BOT, are Unlikely to be Misdiagnosed as Oropharyngeal Squamous Cell Carcinomas

HPV-associated oropharyngeal SCC treated from 1983 to 2013 in the University of Pittsburgh Medical Center and identified as part of a prior project (n = 89) [20] were re-reviewed and the diagnosis of squamous cell carcinoma was confirmed in all cases.

Analysis of Previously Reported Cases

The updated clinical follow-up for the 3 previously reported cases of HPV-associated adenocarcinomas of the BOT is summarized in Table 2. All previously reported cases were negative for *PIK3CA* exon 9 or 20 and *MET* exon 2 mutations.

Fig. 1 Base of tongue biopsy, histologic, immunohistochemical, and in situ hybridization findings. **a** The adenocarcinoma undermines squamous epithelium and is surrounded by the lymphoid stroma of the base of tongue, H&E, $\times 200$. **b** Well-formed glands of adenocarcinoma, H&E, $\times 600$. **c** p16 stain shows strong cytoplasmic and nuclear expression in the majority of neoplastic cells, immunohistochemistry, $\times 100$. **d** HPV in situ hybridization shows punctate nuclear staining, $\times 600$

Table 2 Updated clinical follow-up and *PIK3CA* mutational status of previously reported cases of HPV-associated adenocarcinomas of the base of tongue

Case # and reference	Gender and age (years)	TNM at presentation	<i>PIK3CA</i> status	Treatment	Outcome
1. [10]	Male, 64	pT3 N2b M0	WT	Supraglottic laryngectomy with BOT resection and RT	Alive, NED, 21 months after the diagnosis
2. Case #1 [9]	Male, 57	cT2 NX M0	WT	NA	Alive, NED, 10 months after the diagnosis
3. Case #2 [9]	Male, 53	pT2 N2c M0	WT	BOT resection and CRT	DOD, 37 months after the diagnosis
4. Case presented in this report	Male, 70	cT2 N2b M0	p.E545K	CRT	Interval decrease in tumor size, 5 months after the diagnosis

BOT base of tongue, *WT* wild type, *RT* radiotherapy, *NA* not available, *CRT* chemoradiotherapy, *NED* no evidence of disease, *DOD* died of disease

Discussion

We present an additional case of HPV-associated adenocarcinoma, NOS, of the BOT, along with the updated clinical history and molecular testing of the three previously reported cases [9, 10]. As in prior reports, this adenocarcinoma showed no morphologic or immunophenotypic features of a named salivary type carcinoma. We also considered the possibility of a metastasis to the BOT from an occult distant primary. However, the results of imaging studies and the tumor's immunoprofile argue against this possibility.

As previously suggested, HPV-associated adenocarcinomas, NOS, of the BOT are rare: of the 46 difficult to classify head and neck carcinomas tested by Krane et al all were HPV negative [9]. Here we show that HPV-associated

adenocarcinomas of the BOT are unlikely to be confused with HPV-positive non-keratinizing oropharyngeal SCC.

The increased recognition of HPV-positive adenocarcinomas of the BOT further shows that HPV is associated with a morphologically wide spectrum of oropharyngeal carcinomas [5–8] and the HPV role in oropharynx appears to be similar to that in the uterine cervix [25–27].

For the newly identified case, we have employed an IonTorrent™ semiconductor sequencing technology and direct sequencing to identify and confirm the presence of *PIK3CA* p. E545K and *MET* p.E168D mutations. Of the two identified mutations, *PIK3CA* is of more immediate significance: it was recently shown that HPV-positive SCC tumor grafts with an activating *PIK3CA* mutation were highly responsive to PI3K-targeted therapy [12]. Promising findings have been also reported in clinical trials of patients

with gynecologic malignancies, including uterine cervix [19]. Therefore, the three previously reported cases of HPV-associated adenocarcinomas of the BOT were also tested for *PIK3CA* hotspot mutations by SNaPshot PCR. Altogether, 1 of 4 cases harbored a *PIK3CA* mutation. These molecular findings suggest that for a subset of HPV-associated oropharyngeal adenocarcinomas one may consider therapeutic targeting of the PI3K pathway. The most recent characterization of the mutational profile of head and neck SCC showed that the genetic landscape of HPV-positive SCC is distinct from that of HPV-negative SCC. For instance, HPV-positive oropharyngeal SCCs harbor fewer mutations overall (e.g., no *TP53* mutations, as in our case) and have higher prevalence of *PIK3CA* mutations. Specifically, of the 15 HPV-positive SCC with reported *PIK3CA* status, 4 tumors harbored *PIK3CA* mutation (27 %). [13, 28] While the number of cases we could test is limited by the rarity of HPV-associated adenocarcinomas of BOT, the prevalence of *PIK3CA* mutations (1/4, 25 %) is consistent with that in HPV-positive oropharyngeal SCC.

The *MET* proto-oncogene encodes the receptor tyrosine kinase, the sole ligand for which is hepatocyte growth factor. Most *MET* mutations result in increased MET activity. The MET p.E168D mutation (exon 2, semaphorin domain) is rare and was first reported in a case of small cell lung carcinoma [29] and later identified in a case of head and neck SCC with unspecified clinicopathologic details [30], two cases of undifferentiated carcinoma with unknown primary [31], and one case of Langerhans cell histiocytosis [32]. The *PIK3CA* or HPV status in these cases was unknown.

One of the limitations of this report is the lack of experimental or clinical data on whether *PIK3CA* and/or *MET* mutations are actually oncogenic in the HPV-associated adenocarcinoma of the BOT.

In summary, we describe a rare case of HPV-positive adenocarcinoma of the BOT.

Further studies are needed to truly understand the clinical implications, origin (mucosal vs. salivary), and molecular profile of this tumor.

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