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ORIGINAL PAPER

Mucoepidermoid Carcinoma Does Not Harbor Transcriptionally Active High Risk Human Papillomavirus Even in the Absence of the *MAML2* Translocation

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Abstract High risk human papillomavirus (HPV) is firmly established as an important cause of oropharyngeal carcinoma. Recent studies have also implicated HPV as a cause of mucoepidermoid carcinoma (MEC)—a tumor of salivary gland origin that frequently harbors *MAML2* translocations. The purpose of this study was to determine the prevalence of transcriptionally active HPV in a large group of MECs and to determine whether HPV infection and the *MAML2* translocation are mutually exclusive events. Break-apart fluorescence in situ hybridization for *MAML2* was performed on a tissue microarray containing 92 MECs. HPV testing was performed using RNA in situ hybridization targeting high risk HPV mRNA E6/E7 transcripts. Of the 71 MECs that could be evaluated by FISH, 57 (80 %) harbored the *MAML2* rearrangement. HPV was not detected in any of the 57 MECs that contained a *MAML2* rearrangement, in any of the 14 MECs that did not contain the rearrangement, or in any of the 21 MECs where *MAML2* status was unknown. High risk HPV does not appear to play any significant role in the development of MEC. It neither complements nor replaces *MAML2* translocation in the tumorigenesis of MEC.

Keywords Mucoepidermoid carcinoma · Human papillomavirus · *MAML2*

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Introduction

Human papillomavirus (HPV) is now well established as an important cause of head and neck cancer [1–3], but its distribution is highly restricted by anatomic site and tumor type. It is detected in 50–80 % of oropharyngeal cancers where it tracks with the non-keratinizing squamous cell phenotype, but it is not frequently detected in head and neck squamous cell carcinomas arising outside of the oropharynx [4–7]. In oropharyngeal carcinomas, the detection of HPV is of great clinical significance, as HPV positivity is associated with improved clinical outcomes in ways that modulate therapeutic management [8, 9]. In view of its profound clinical relevance as a biomarker for patients with oropharyngeal squamous cell carcinoma, there is considerable interest in identifying high risk HPV in other types of head and neck cancer.

Mucoepidermoid carcinoma (MEC) is the most common type of salivary gland carcinoma. Up to 75 % of MECs harbor chromosomal rearrangements involving *MAML2* [10–12]. Some have suggested that the presence of a *MAML2* rearrangement identifies a biologically distinct group of MEC with a less aggressive clinical behavior, but other molecular genetic factors that act in concert with or independent of the *MAML2* rearrangement are not yet well defined. Recently, high risk HPV has been identified in a significant subset of MECs [13]. We performed RNA

in situ hybridization for E6/E7 mRNA transcripts both to confirm the high prevalence of transcriptionally active HPV in MECs and to determine the relationship between HPV infection and *MAML2* translocation.

Methods

Cases

This study was approved by Institutional Review Board of The Johns Hopkins Medical Institutions. The surgical pathology files of The Johns Hopkins Hospital were searched for cases of MEC diagnosed from 1984 to 2012. Hematoxylin and eosin-stained sections were reviewed to confirm the diagnosis, and the tumors were graded using the grading scheme advocated by the World Health Organization [14].

Tissue Microarray

A tissue microarray (TMA) was constructed from the formalin-fixed paraffin-embedded (FFPE) tissue blocks of 92 MECs. The MECs consisted of 45 low grade, 29 intermediate grade, and 18 high grade carcinomas. The primary sites were the parotid gland (n = 43), oral cavity (n = 39), submandibular gland (n = 6) and sinonasal tract (n = 4). Three cores, each 1 mm in diameter, were taken from each donor block to address tumor heterogeneity.

MAML2 Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) was performed on FFPE section using a commercially available *MAML2* dual color break apart probe (Z-2014-200, Zytovision, Germany). Prior to hybridization, the slides were deparaffinized using a VP 2000 processor (Abbott Molecular, Des Plains, IL, USA) in which pretreatment with protease I was used. Following deparaffinization, the slides and the *MAML2* probe were co-denatured at 80 °C for 7 min and allowed to hybridize for 22 h at 37 °C in humidified atmosphere. At the end of the incubation, the slides were washed in 2 × SSC/0.3 % NP-40 for 2 min at 72 °C and for 2 min at room temperature, with agitation. Traces of detergent were removed by washing the slides in 2 × SSC at room temperature. The slides were counterstained with DAPI, and a cover slip was applied using Vectashield mounting medium (H-1000, Vector Laboratories, Inc.). A fluorescence microscope was used to evaluate the probe pattern. Cells with two fusion signals of one orange and one green fluorochrome were scored as normal. Cells with rearrangements for *MAML2* gene had one normal fusion signal and one orange and one green signal at a distance from each other. A mucoepidermoid carcinoma known to harbor the *MAML2* rearrangement served as a positive control, while normal salivary tissue served as a negative control.

HPV RNA In Situ Hybridization

HPV status was determined using an RNA in situ hybridization approach. In this study, p16 immunohistochemical staining was not used because p16 staining has been found to be a very poor surrogate marker for the presence of high risk HPV when dealing with salivary gland tumors [15, 16]. In situ hybridization for HPV E6/E7 mRNA was performed manually using the RNAscope kit (Advanced Cell Diagnostics Inc., Hayward, CA, USA). The HPV HR (18) probe containing a cocktail of 18 high-risk HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) was used for HPV detection. A probe to the housekeeping gene PPiB (Peptidyl-prolyl cis–trans isomerase B) was used as a positive control to ensure presence of an intact RNA target in the specimen, and a known HPV-related squamous cell carcinoma included on the TMA to serve as a positive control (Fig. 1). A probe to the bacterial gene dapB was used as a negative control. The staining protocol was carried out according to the manufacturer's instructions. The incubations/hybridization steps were performed in HybEZ^{TM} Oven, which provides a gasket-sealed, temperature-controlled humidifying chamber (Advanced Cell Diagnostics Inc., Hayward, CA). Briefly, 4-mm-thick FFPE tissue sections were pretreated with heat (slow-boiling in P2 solution for 10 min) and protease (P3, 1:5 dilution, at 40 °C in the humid chamber) before probe hybridization. The preamplifier, amplifier, and horseradish peroxidase-labeled probes were then hybridized sequentially, followed by color development with diaminobenzidine. Specific staining signals were identified as brown, punctate dots present in the cytoplasm and/or nucleus.

Results

The status of the MECs for the presence of the *MAML2* rearrangement and high risk HPV infection as a function of tumor grade and tumor site are summarized in Table 1. Break-apart FISH for the *MAML2* rearrangement was successfully performed on 71 of 92 (77 %) MECs. 57 of 71 (80 %) were positive for the rearrangement including 37 of 42 (88 %) low grade, 17 of 20 (85 %) intermediate grade, and 3 of 9 (33 %) high grade MECs. Low and intermediate grade MECs were statistically more likely to harbor the *MAML2* rearrangement than high grade MECs ($p = 0.001$, Fisher's exact test, 2-sided). By anatomic site, the *MAML2*

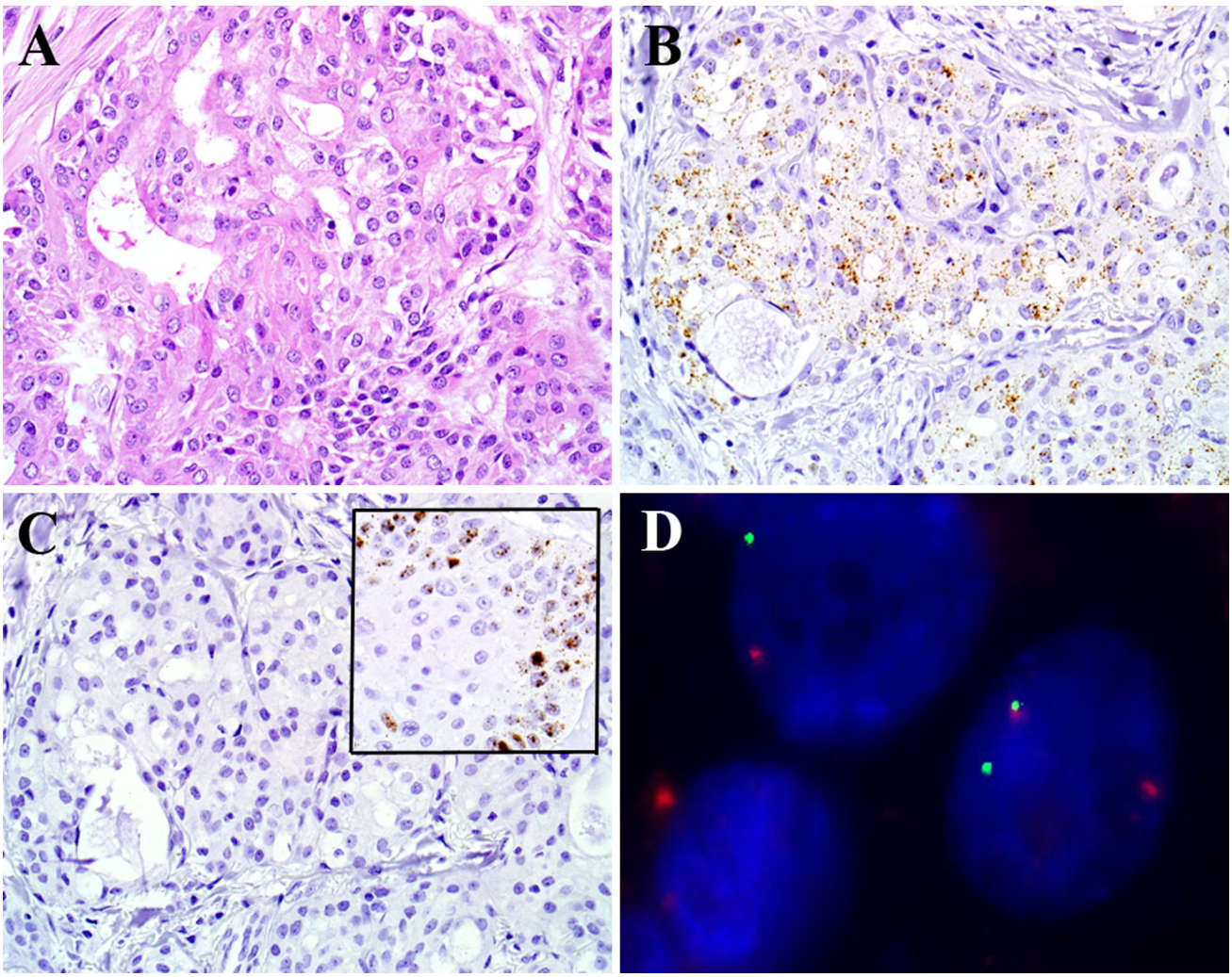

Fig. 1 By RNA in situ hybridization, this example of mucoepidermoid carcinoma (**a**, hematoxylin and eosin stain) was positive for transcripts of the housekeeping gene peptidyl-prolyl isomerase B (**b**, RNA in situ hybridization) but completely negative for the E6 and E7 viral transcripts of high risk HPV (**c**, RNA in situ hybridization; *inset* showing positive hybridization signals in an HPV-positive oropharyngeal carcinoma serving as a positive control). Break apart fluorescent in situ hybridization for *MAML2* was positive (separate green and *red* signals) (**d**, fluorescent in situ hybridization)

rearrangement was detected in 31 of 39 (79 %) parotid tumors, 22 of 26 (85 %) oral cavity tumors, 3 of 4 (75 %) submandibular tumors, and 1 of 2 (50 %) sinonasal tumors. High risk HPV was not detected in any of the 92 MECs by RNA in situ hybridization (Fig. 1).

Discussion

In the oropharynx, the recognition of an HPV-related form of oropharyngeal cancer has opened the door to a new understanding of tumorigenesis that is translating into effective prevention measures (e.g. preventive vaccines) and novel therapeutic strategies (e.g. therapeutic vaccines and de-intensification therapy). Recent studies have suggested that, like the oropharynx, the salivary glands may also be targeted by oncogenic HPV [13, 16–18]. These HPV-related forms of salivary gland cancer warrant considerable attention as they could provide new insights into a type of head and neck cancer in which little is known about the biologic mechanisms underlying tumorigenesis.

In light of potential clinical repercussions, claims regarding the identification of a new form of HPV-related head and neck cancer warrant careful corroboration. For example, the existence of a newly identified HPV-related adenoid cystic carcinoma either has been unconfirmed in subsequent studies or has been redefined as non-salivary gland carcinoma [5, 16, 19, 20]. Recently, Isayeva et al. [13]. reported that about half of all MECs are infected with transcriptionally active high risk HPV, an incidence that rivals its occurrence in the oropharynx.

Table 1 *MAML2* translocation status and HPV status in mucoepidermoid carcinomas as a function of tumor grade and site

Mucoepidermoid carcinoma	*MAML2* translocation (n = 71)		High risk HPV (n = 92)	
	Present (%)	Absent (%)	Positive (%)	Negative (%)
Grade				
Low	37 (88)	5 (12)	0 (0)	45 (100)
Intermediate	17 (77)	3 (23)	0 (0)	29 (100)
High	3 (33)	6 (67)	0 (0)	18 (100)
Site				
Parotid gland	31 (79)	8 (21)	0 (0)	43 (100)
Oral cavity	22 (85)	4 (15)	0 (0)	39 (100)
Submandibular gland	3 (75)	1 (25)	0 (0)	6 (100)
Sinonasal tract	1 (50)	1 (50)	0 (0)	4 (100)
Total	57 (80)	14 (20)	0 (0)	92 (100)

In an effort to substantiate this finding, Jour et al. [15] were not able to detect HPV in any of their MECs, but their number of cases was limited (n = 14) and HPV detection was restricted to a DNA in situ hybridization approach. Our study was designed to help resolve the uncertainty surrounding the potential role of HPV in MECs. First, we analyzed a large group of MECs across all histologic grades. With MECs in particular, the genetic profile may modulate tumor grade in ways where certain alterations may elude detection if case selection is too restricted. As one example, the *MAML2* rearrangement is known to be much more common in low grade than high grade MECs [12, 21, 22]. Second, our analysis included documentation of *MAML2* status. As *MAML2* translocation resulting in NOTCH pathway activation is believed to drive oncogenic transformation [23], its presence could preclude HPV-induced oncogenesis in much the same way that p53 inactivation and HPV infection are inversely proportional in head and neck squamous cell carcinomas [24–26]. Awareness of *MAML2* status could potentially uncover bias based on overrepresentation of translocation positive tumors. Third, this study takes advantage of recently developed RNA in situ hybridization probes complementary to E6/E7 mRNA that permit direct visualization of viral transcripts in routinely processed tissues. In formalin-fixed and paraffin-embedded oropharyngeal carcinomas, the sensitivity of this method has been shown to exceed that of HPV DNA in situ hybridization [6, 27–29].

Using the RNA in situ hybridization approach, we found that transcriptionally active HPV is not commonly encountered in MECs. Indeed, E6/E7 mRNA viral transcripts were not detected in any of the 92 MECs evaluated including those 14 tumors that were known not to harbor a driver translocation involving *MAML2*. The absence of high risk HPV using an RNA in situ hybridization approach concurs with its reported absence in those studies using a DNA in situ hybridization approach [15, 19]. Outlier studies may reflect, in part, differences in the ability to distinguish between biologically relevant and irrelevant HPV based on methodologies and test interpretation. For example, Isayeva et al. [13]. used highly sensitive PCR-based methods to detect HPV E6/E7 mRNA transcripts in 43 % of MECs, but the presence of these transcripts did not correlate with overexpression of $P16^{INK4a}$—a marker that is now widely used to confirm both the presence and biologic activity of HPV in oropharyngeal carcinomas [29]. HPV DNA in situ hybridization was also used by the Isayeva group to confirm the presence of integrated virus, but the interpretation of cytoplasmic hybridization signals as evidence of transcriptionally active HPV represents a deviation from the standard practice where only nuclear signals are regarded as positive (http://www.uclad.com/newsletters/HPV_ISH_Tissue-Probe-Interpretation_Guide.pdf).

Based on our findings, oncogenic forms of HPV do not appear to be a relevant cause of MEC, either as a primary agent or as a substitutionary agent in those MECs lacking a driver *MAML2* translocation. This observation may appropriately temper recent enthusiasm for exploiting HPV detection as a relevant biomarker when dealing with MECs. Indeed, the finding that the *MAML2* translocation correlates with tumor grade supports the view that *MAML2* status, not HPV status, may prove to be a much more promising prognostic marker for patients with MEC [12, 21, 22].

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