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Clear Cell Carcinoma of Salivary Glands is Frequently p16-Positive: A Pitfall in the Interpretation of Oropharyngeal Biopsies

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Abstract

Clear cell carcinoma (CCC) is a low-grade malignancy that commonly arises in minor salivary glands of the oropharynx and other sites. *EWSR1-ATF1* gene fusions appear to be specific for this salivary neoplasm. Testing for *EWSR1-ATF1* has expanded the histologic spectrum of CCC. As one important example, many CCCs have a predominantly squamous phenotype with few clear cells, a finding that can cause confusion with squamous cell carcinoma (SqCC). P16 immunohistochemical staining to determine HPV status has become standard practice for all oropharyngeal carcinomas showing squamous differentiation. The purpose of this study was to determine whether this practice could contribute to the difficulty in distinguishing CCC from p16-positive SqCC.

The authors' surgical pathology archives were searched for cases of CCC. All cases were evaluated with p16 immunohistochemistry, high risk HPV RNA in situ hybridization (ISH), and *EWSR1* gene break-apart fluorescence ISH.

Sixteen CCCs were identified. All harbored an *EWSR1* rearrangement. Eleven patients were women and 5 were men. They ranged in age from 30 to 85 years (mean, 58). The CCCs arose in the oropharynx (tongue base or tonsil) (n=8, 50%), oral cavity (n=4, 25%), and nasopharynx (n=4, 25%). Each case demonstrated clear cells, but the proportion was highly variable (10 to 90%, mean 48%), with 7 of 16 cases having <50% clear cells. Submitted diagnoses included SqCC (n=3) and mucoepidermoid carcinoma (n=2). Of the 3 patients diagnosed with SqCC, 1 was scheduled to undergo chemoradiation, and 1 had already completed chemoradiation. All 16 CCCs demonstrated p16 staining, with the percentage of p16-positive cells ranging from 70% (n=2), 50–69% (n=3) and 10–49% (n=11). Staining was cytoplasmic and nuclear. All cases were negative for high-risk HPV by RNA ISH.

CCCs regularly show squamous features, often lack prominent clear cell changes, frequently arise in the oropharynx, and invariably show p16 staining. These features may cause confusion with SqCC, particularly HPV-related oropharyngeal SqCC. P16 staining is not to be taken as unequivocal evidence of an HPV-related SqCC, even for carcinomas showing squamous

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differentiation and originating in the oropharynx. Failure to recognize this pitfall could result in overly aggressive treatment of a low-grade carcinoma.

Keywords

clear cell carcinoma; hyalinizing clear cell carcinoma; p16; *EWSRI-ATFI*; human papillomavirus; HPV

Introduction

Clear cell carcinoma (CCC) is a low-grade salivary gland malignancy that was initially described in 1994.(1) While it may occur in the major salivary glands, CCC most commonly arises in the minor salivary glands at the base of tongue or palate. Until recently, CCC was regarded not as a distinct entity but rather as a diagnosis of exclusion – the default diagnosis for any clear cell-predominant salivary gland carcinoma that failed to meet criteria for some more specific tumor type such as epithelial-myoepithelial carcinoma, myoepithelial carcinoma or mucoepidermoid carcinoma. Indecisiveness about the true nature and identity of CCC is reflected in the ever-evolving terminology used to properly designate it. Originally designated as “hyalinizing clear cell carcinoma,”(1) it was renamed as “clear cell carcinoma, not otherwise specified” in the 2005 World Health Organization Classification (WHO) of Head and Neck Tumors,(2) then as “clear cell adenocarcinoma” in the 2008 Armed Forces Institute of Pathology Fascicle of Salivary Gland Tumors,(3) and now simply as “clear cell carcinoma” in the updated 2017 WHO Classification of Head and Neck Tumors.(4) The notion that CCC is nothing more than a mishmash of salivary gland tumors linked together by the presence of non-descript clear cells has been recently dismissed by Antonescu et al.(5) They found that that most CCCs harbor *EWSRI-ATFI* fusions not found in any other types of salivary gland tumors, supporting the verdict that CCC is a distinct tumor entity.(5) The presence of a diagnostic molecular marker for CCC has now allowed for a more complete appreciation of its full histologic spectrum that, in turn, has exposed a vulnerability in diagnosing CCC based on histology alone. For example, CCCs sometimes exhibit overt squamous differentiation, they often are not hyalinized, and they may not even be dominated by clear cells. For all of these reasons, CCCs run the risk of being misdiagnosed as high-grade squamous cell carcinomas (SqCC), particularly when they arise from minor salivary glands of the oropharynx and other mucosal sites.

A majority of SqCCs arising in the oropharynx are now known to harbor high-risk human papillomavirus (HPV), and these HPV-related oropharyngeal SqCCs have a significantly improved survival and response to therapy compared to HPV-unrelated SqCC.(6, 7) As a result, routine HPV testing is now universally recommended for all newly diagnosed SqCCs of the oropharynx.(8–10) While several HPV testing methods are available, p16 immunohistochemistry is now accepted as a reliable standalone test for determining the HPV status oropharyngeal SqCCs.(9, 10) For SqCCs arising within the oropharynx, p16 staining is highly sensitive and specific such that p16 staining has become the diagnostic equivalent of HPV-positive SqCC. Few if any pitfalls associated with positive p16 staining in oropharyngeal carcinomas are currently recognized. The purpose of this study was to

establish the p16 status of CCCs in an effort to determine whether p16 staining would clarify or confound its distinction from SqCC, particularly for those tumors arising in the oropharynx.

Materials and Methods

Cases

With IRB approval (0001336), cases of CCC were retrieved from the authors' surgical pathology archives including their consultation files. Basic demographic information, tumor location, and submitted diagnosis (if any) were recorded. All cases were reviewed centrally by two study pathologists (JAB, WHW) to confirm the diagnosis and document the proportion of clear cells within the tumor.

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was performed on formalin fixed paraffin embedded (FFPE) sections using a commercially available *EWSR1* dual color break apart probe (ZTV-Z-2096-200, ZytoVision, Germany). The probe is designed to detect translocations involving the chromosomal region 22q12.2 harboring the *EWSR1* gene. Prior to hybridization the slides were deparaffinized using a VP 2000 processor (Abbott Molecular, Des Plaines, IL). The slides and the *EWSR1* probe were co-denatured at 80 °C for 7 minutes and allowed to anneal over night at 37 °C in humidified atmosphere. At the end of the incubation the slides were washed in $2 \times \text{SSC}/0.3\% \text{ NP-40}$ for 2 min at 72 °C and for 2 min at room temperature in $2 \times \text{SSC}$. 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, and considered negative for *EWSR1* rearrangement. A signal pattern consisting of one fusion signal and one orange and one green signal, at distance from each other, indicates one normal 22q12.2 locus and one locus affected by translocation.

Immunohistochemistry

P16 (clone INK4a; MTM Laboratories, Heidelberg, Germany; prediluted by manufacturer) immunohistochemical studies were performed on 5 µm-thick sections prepared from formalin-fixed and paraffin-embedded tissue using standard autostaining protocols on a Ventana Benchmark XT autostainer (Ventana Medical Systems, Inc, Tucson, AZ). Deparaffinization and antigen retrieval (i-view detection system; Ventana) were carried out as an automated program of the Ventana autostainer. The extent (% tumor cells) and distribution (nuclear and/or cytoplasmic) of staining was recorded.

Human Papillomavirus RNA In Situ Hybridization

Sections of 5 µm from formalin-fixed and paraffin-embedded tissue blocks were evaluated for the presence of HPV RNA using a manual RNAscope HPV-HR18 Probe (Advanced Cell Diagnostics, Hayward, CA) which recognizes 18 high-risk HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82). HPV positive controls included HPV 16-positive head and neck squamous cell carcinoma as well as the HPV 16-positive

SiHa and CaSki cell lines. An HPV-negative head and neck squamous cell carcinoma served as a negative control.

Results

The results are summarized in Table 1. Sixteen CCCs were identified including 4 routine and 12 consult cases. Each case harbored a *EWSR1* gene fusion as demonstrated by FISH (Figure 1 **inset**). They occurred in 11 women and 5 men ranging in age from 30 to 85 years (mean, 58 years). Eight (50%) arose in the oropharynx (7 base of tongue and 1 tonsil), 4 (25%) in the oral cavity (1 retromolar trigone, 1 gingiva, 1 hard and soft palate, and 1 oral tongue), and 4 (25%) in the nasopharynx. Each case demonstrated some cells with clear cytoplasm, but the proportion was highly variable, ranging from 10 to 90% clear cells (mean 48%) with 7 of 16 cases having <50% clear cells (Figure 1A–C and Figure 2A). In 4 of 16 cases the CCC exhibited surface epithelial involvement (Figure 1A) but squamous dysplasia/carcinoma in situ was not identified.

All 16 cases showed some immunoreactivity for p16. The extent of staining was variable, ranging from 5 to 80% of cells (mean, 30%). P16 staining was seen in 70% of cells in 2 cases, 50–69% of cells in 3 cases, and 10–49% of cells in 11 cases. In all cases, the p16 immunostaining was nuclear and cytoplasmic, with a nuclear predominance (Figure 2B). All cases were negative for high-risk HPV by RNA in situ hybridization (Figure 2B **inset**).

Twelve of 16 cases were submitted with a final or preliminary diagnosis from the contributing pathologist, including SqCC (n=3), CCC (n=3), mucoepidermoid carcinoma (n=2), low-grade carcinoma with clear cells cannot exclude SqCC (n=1), salivary carcinoma not otherwise specified cannot exclude SqCC (n=1), low-grade adenocarcinoma not otherwise specified (n=1), and carcinoma with clear cell features (n=1). One of the patients originally diagnosed as SqCC was scheduled to undergo chemoradiation, and another patient diagnosed as p16-positive oropharyngeal SqCC had already completed chemoradiation but with no tumor response.

Discussion

Over the past several years, an expanding list of salivary gland neoplasms has been shown to harbor characteristic gene fusions. Clear cell carcinoma (*EWSR1-ATF1*), (5) mucoepidermoid carcinoma (*CRTC1-MAML2* or *CRTC3-MAML2*), (11, 12) adenoid cystic carcinoma (*MYB-NFIB* or *MYBL1-NFIB*), (13, 14) secretory carcinoma (*ETV6-NTRK3*), (15) and polymorphous adenocarcinoma (*PRKDI-3* partnered with various genes) (16) are now known to harbor tumor-specific gene fusions. The presence of these recurring tumor-specific gene fusions has given pathologists a diagnostic benchmark for tumor classification that has, in turn, facilitated an appreciation for phenotypic divergence within salivary gland tumor types. For example, the detection of *MAML2* rearrangements in Warthin-like and oncocytic neoplasms has confirmed these as phenotypic variants of mucoepidermoid carcinomas. (17, 18) Along these same lines, the discovery of tumor-specific *EWSR1-ATF1* fusions in CCC has expanded the range of its histologic characteristics. It is now recognized that CCCs sometimes lack a predominance of clear cells, involve the surface epithelium,

demonstrate well-developed squamous features, and even produce mucin. Not surprisingly, CCCs run the risk of being misdiagnosed as either SqCC or mucoepidermoid carcinoma when tumor classification is based on morphologic features alone.(19, 20) Indeed, in our limited series of CCCs, 7 were misdiagnosed by the submitting pathologist as either SqCC (n=3), cannot exclude SqCC (n=2), or mucoepidermoid carcinoma (n=2). For infiltrative carcinomas of the head and neck that show squamous differentiation, the differential diagnosis should not invariably be restricted to SqCC, especially for those tumors that lack conventional surface dysplasia and overt keratinization, demonstrate a more uniform cell population without high-grade features, and exhibit varying degrees of cytoplasmic clearing and stromal hyalinization. Some of these may represent CCCs and require confirmative *EWSR1-ATF1* testing.

The propensity of CCC to be misclassified as SqCC may be exacerbated in the oropharynx, a site where carcinomas showing squamous differentiation routinely undergo testing to determine HPV status. The confusion may also extend to the nasopharynx where 25% of our CCCs occurred and where emerging evidence suggests HPV-related SqCC also arise and are typically treated non-surgically.(21, 22) We found that CCC commonly demonstrates p16 positivity, even in a diffuse pattern and with a nuclear and cytoplasmic distribution - a pattern and distribution that has been tightly correlated with integrated high-risk HPV. But even though all of the CCCs demonstrated some degree of p16 staining, none were found to harbor transcriptionally-active high-risk HPV. This dichotomy highlights the danger in accepting p16 staining alone as unequivocal evidence of an HPV-related carcinoma, even when dealing with oropharyngeal carcinomas showing squamous differentiation.

Although confusing CCC with HPV-related SqCC is understandable, it is a significant diagnostic error that carries serious clinical consequences. CCC is a low-grade carcinoma that is readily cured by surgical excision. HPV-related SqCC, on the other hand, is high-grade carcinoma that is often treated by various therapeutic modalities including radiation therapy with or without chemotherapy. Two of the study patients had or were about to receive chemoradiation based on an erroneous diagnosis of SqCC, even though CCCs are not known to respond to SqCC chemotherapy protocols. The future use of HPV-specific therapies such as therapeutic vaccines only further underscores the need to clearly separate CCC from HPV-SqCC for oropharyngeal carcinomas in the oropharynx showing squamous differentiation and p16 positivity.(23)

Recognition of the pattern of p16 immunostaining is crucial to its interpretation as a surrogate HPV marker. According to recent College of American Pathologists guidelines, to regard an oropharyngeal carcinoma as p16-positive, 70% of cells must be positive, staining should be both nuclear and cytoplasmic, and moderate to strong in intensity.(10) For SCC not meeting these criteria, p16 should be regarded as either negative (<50% staining), or equivocal (>50% but <70% staining, or >70% but weak intensity).(10) Close attention to these criteria is useful in avoiding the pitfall of diagnosing p16-positive CCC as HPV-related SCC. While all CCCs demonstrated some p16 immunolabeling, only 2 of 12 cases were positive in 70 of cells and would have been called positive by these criteria.

In conclusion, CCCs frequently arise in the oropharynx, often show well-developed squamous features, often lack prominent clear cell features, and are invariably p16-positive. This profile may cause confusion with SCC in general, and with HPV-related oropharyngeal SCC in particular, with significant clinical consequences. As a result, p16 staining by itself should not be taken as unequivocal evidence of an HPV-related SCC, even for carcinomas showing squamous differentiation and originating in the oropharynx.

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Figure 1.

A, Clear cell carcinoma frequently demonstrates surface epithelial involvement and an eosinophilic appearance, features that overlap with squamous cell carcinoma. B, Clear cell carcinoma may also exhibit overt keratinization (arrows). C, More classically, clear cell carcinoma is comprised of nests and cords of bland cells with clear cytoplasm surrounded by hyalinized fibrosis. Most cases of clear cell carcinoma harbor *EWSR1* rearrangements, demonstrated by break apart fluorescence in situ hybridization (inset).

Figure 2.

A, Clear cell carcinoma with a mix of eosinophilic and clear cells. B, CCC was variably positive for p16 by immunohistochemistry with a cytoplasmic and nuclear pattern. All cases, however, were negative for high-risk human papillomavirus by RNA in situ hybridization (inset).

Table 1

Clinicopathologic characteristics of hyalinizing clear cell carcinomas.

Case #	Age	Sex	Location	p16 IHC ¹ (%)	Clear Cells (%)	<i>EWSR1</i> FISH ²	Submitted Diagnosis
1	67	M ³	Base of tongue	5	30	+	Squamous cell carcinoma
2	50	F ⁴	Base of tongue	5	80	+	Mucoepidermoid carcinoma
3	43	M	Base of tongue	50	20	+	None
4	47	F	Base of tongue	60	50	+	Squamous cell carcinoma
5	78	F	Base of tongue	80	50	+	Clear cell carcinoma
6	71	F	Base of tongue	10	50	+	None
7	59	M	Base of tongue	70	10	+	Squamous cell carcinoma
8	69	M	Tonsil	30	20	+	Low grade carcinoma with clear cells, cannot exclude squamous cell carcinoma
9	45	F	Nasopharynx	20	20	+	Mucoepidermoid carcinoma
10	66	F	Nasopharynx	50	80	+	Clear cell carcinoma
11	30	F	Nasopharynx	5	30	+	Salivary carcinoma, NOS, ⁵ cannot exclude squamous cell carcinoma
12	67	F	Nasopharynx	10	80	+	Low grade adenocarcinoma, NOS
13	40	F	Retromolar trigone	20	50	+	Carcinoma with clear cell features
14	31	M	Gingiva	5	30	+	Clear cell carcinoma
15	85	F	Hard and soft palate	30	80	+	None
16	80	F	Tongue	30	90	+	None

¹IHC = immunohistochemistry;

²FISH = fluorescence in situ hybridization;

³M = male;

⁴F = female;

⁵NOS= not otherwise specified.