Spindle Cell Carcinomas of the Head and Neck Rarely Harbor Transcriptionally-Active Human Papillomavirus

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Received: 28 December 2012 / Accepted: 22 March 2013 / Published online: 28 March 2013
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Abstract Spindle cell carcinoma is an uncommon variant of squamous cell carcinoma characterized by spindled or pleomorphic cells which appear to be a true sarcoma but are actually epithelial. Some head and neck squamous cell carcinoma variants can be human papillomavirus (HPV)-related and have improved outcomes. We sought to determine if spindle cell carcinomas are associated with transcriptionally-active HPV. Cases of spindle cell carcinoma were retrieved from department files. Transcriptionally-active HPV was determined by mRNA in situ hybridization for high risk HPV E6 and E7 transcripts and by a surrogate marker, p16 immunohistochemistry, with a 50 % staining cutoff. RT-PCR for high risk HPV mRNA was performed on the cases that were technical failures by in situ hybridization. Medical records and follow up

information were retrieved for all patients. Of 31 cases, 5 were from the oropharynx, 12 from the oral cavity, and 14 from the larynx or hypopharynx. One purely spindled oral cavity spindle cell carcinoma was HPV positive. It was also diffusely positive for p16. Another laryngeal spindle cell carcinoma was HPV positive in both the squamous and spindle cell components, but was negative for p16. None of the five oropharyngeal spindle cell carcinomas were positive for p16 or HPV RNA. The HPV positive patients both presented at high stage (IV) and died with disease within 2 years of diagnosis. The majority of spindle cell carcinomas of the head and neck, including those arising in the oropharynx, are not related to transcriptionally active HPV. Although the number of cases is too small for any definitive conclusions, for the rare HPV positive spindle cell carcinoma cases, positive viral status does not appear to confer any prognostic benefit.

Presented at the United States and Canadian Academy of Pathology, 101st annual meeting, Vancouver, BC, Canada, 2012.

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Keywords Spindle cell carcinoma · Sarcomatoid carcinoma · Human papillomavirus · RNA · p16 immunohistochemistry · In situ hybridization

Introduction

Spindle cell carcinoma is an uncommon variant of squamous cell carcinoma that occurs in several subsites in the head and neck [1–4]. These tumors are histologically diverse and are classically characterized by a biphasic morphologic appearance with both sarcomatoid (spindle cell) and conventional squamous cell carcinoma components [5]. A significant minority of spindle cell carcinomas consist only of a spindle cell or sarcomatoid component. These cases, in particular, can be diagnostically challenging. Immunohistochemistry, especially for the epithelial

markers p63 and pan-cytokeratin, can be very useful in distinguishing spindle cell carcinomas from true sarcomas [4, 6, 7]. Interestingly, spindle cell carcinomas also have a unique macroscopic appearance, frequently presenting as polypoid, exophytic masses. Spindle cell carcinomas have similar demographics to conventional squamous cell carcinoma [1, 3, 4, 7, 8]. Besides their relation to heavy smoking and drinking, spindle cell carcinomas frequently occur after radiation for other diseases, at a much higher rate than conventional squamous cell carcinoma [1–4, 6, 7]. Although survival is difficult to study since these are uncommon tumors, the literature suggests a similar overall prognosis to conventional squamous cell carcinoma. However, it appears that while early, polypoid spindle cell carcinomas do well clinically, those presenting at higher stages may do worse than conventional squamous cell carcinoma when compared stage for stage [7, 8].

In the past, there have been numerous theories about the pathophysiology of spindle cell carcinomas such as that they represent de-differentiation or alternate differentiation of one clone of tumor cells into two distinct histologic populations, that they are “collision” lesions of two completely different tumor clones, or that the spindle cell population just represents a reactive process secondary to the primary carcinoma. Molecular, ultrastructural, and morphologic studies have clearly established that the spindle cell/sarcomatoid component of spindle cell carcinomas is derived from the epithelial component and represents divergent differentiation [4, 9–12].

High risk human papillomavirus (HPV) has become well-established as a causative agent with prognostic implications in the majority of oropharyngeal squamous cell carcinomas and in a minority of squamous cell carcinomas from other head and neck subsites. This appears to be an alternate carcinogenic pathway that requires the suppression of p53 and retinoblastoma protein by the viral oncoproteins E6 and E7 [13, 14]. Numerous retrospective and prospective studies have shown HPV-related oropharyngeal squamous cell carcinomas to have improved prognosis [14–16] and that HPV is an independent prognostic factor. However, HPV must be transcriptionally-active to be clinically relevant [17], and p16 protein, which is extensively overexpressed in tumors with transcriptionally-active HPV, has become an acceptable surrogate marker in oropharyngeal squamous cell carcinoma for the virus and for prognostication [18–20]. HPV DNA can be detected by polymerase chain reaction in a significant fraction of squamous cell carcinomas across all head and neck subsites. However, improved patient outcomes are only seen when it is shown to have transcriptional activity (in other words, with detectable HPV E6 and E7 mRNA).

Although most of the current data on HPV is in conventional squamous cell carcinoma, several studies of squamous cell carcinoma variants such as basaloid

squamous cell carcinoma, adenosquamous carcinoma, neuroendocrine/small cell carcinoma, and papillary squamous cell carcinoma have shown that variable numbers of these tumors, particularly those arising in the oropharynx, have transcriptionally-active HPV [21]. Further, in these variants, with the exception of high grade neuroendocrine/small cell carcinomas, the presence of virus appears to correlate with improved survival, thus “trumping” the morphology [22–24].

To date, there are no publications in the literature on transcriptionally-active HPV in spindle cell carcinomas of the head and neck. However, an abstract presented at the annual USCAP meeting in 2011 suggested that some spindle cell carcinomas are associated with transcriptionally-active HPV [25]. In the current study, we evaluated a series of head and neck spindle cell carcinomas for HPV both directly by assessing for the presence of high risk HPV E6/E7 mRNA by RNA in situ hybridization or RT-PCR and indirectly by assessing for high level p16 protein expression.

Materials and Methods

Study Population

This study was approved by the Human Research Protection Office of Washington University. The pathology files of Barnes-Jewish Hospital were searched from 1988 to 2009 for cases of head and neck carcinomas diagnosed as “spindle cell carcinoma”, “sarcomatoid carcinoma,” or “squamous cell carcinoma with a spindle cell component.” All biopsy and/or surgical resection specimen slides were reviewed by two study pathologists (RFW and JSL). The diagnosis was also independently confirmed by review of representative slides by two other study pathologists (RDC and SEM), all with subspecialty expertise in head and neck pathology. Cases were included only if they met the current 2005 WHO definition [5]. Specifically, cases had to either be biphasic with both conventional squamous cell carcinoma and spindle cell components or monophasic with demonstration of epithelial differentiation by immunohistochemistry. Monophasic cases had to have positivity for at least one epithelial marker, either pan-cytokeratin (AE1/AE3), epithelial membrane antigen, or p63, performed in the initial workup of the case. No additional immunohistochemistry for epithelial differentiation was performed as part of this study.

A small proportion of cases were excluded because immunohistochemistry for epithelial markers was negative. Clinical follow up including overall survival, disease free survival, and treatment data were obtained either through review of patients’ paper or electronic medical records.

p16 Immunohistochemistry

Immunohistochemistry was performed on 4 µm sections from formalin-fixed, paraffin-embedded tissue blocks on a Ventana Benchmark XT automated immunostainer (Ventana Medical Systems, Inc., Tucson, AZ) using standard established laboratory protocols with a monoclonal antibody to p16 (MTM Laboratories; clone E6H4; monoclonal; prediluted). Ventana's ultraView Universal DAB Detection Kit was used as the secondary antibody cocktail to detect binding of the primary antibody and this complex was subsequently visualized using hydrogen peroxide substrate and a 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen. Manufacturer antigen retrieval conditions were used (Ventana CC1, EDTA-Tris, pH 8.0 solution). A known p16 positive oropharyngeal squamous cell carcinoma was used as a positive control. p16 staining had to be both nuclear and cytoplasmic, and the amount of staining was assessed in quartiles as follows: 0 = negative, 1+ = 1–25 % of cells positive, 2+ = 26–50 % of cell positive, 3+ = 51–75 % of cells positive, and 4+ = 76–100 % of cells positive. For study purposes, cases were considered p16 positive if there was at least 50 % staining (3+ or 4+). All p16 immunostains were reviewed by three of the study pathologists (RFW, JSL, and RDC) and discrepancies were resolved by consensus review.

HPV RNA In Situ Hybridization

In situ hybridization for high risk HPV E6/E7 RNA transcripts was performed by hand using the ultra sensitive RNAscope™ HPV kit (Advanced Cell Diagnostics, Inc., Hayward, CA) according to manufacturer instructions. Target probes for multiple HPV high risk genotypes were used (including 16, 18, 31, 33, 35, 52, and 58). First, 4 µm tissue sections were pre-treated with heat and protease digestion before hybridization by the target probes. The signal was then amplified using a horseradish peroxidase-based system and detected using DAB. Probes for the bacterial gene (DapB) and housekeeping gene ubiquitin C served as controls for nonspecific staining and for RNA quality/adequacy, respectively. Cases were classified in a binary fashion as positive or negative based on the presence of brown, punctate spots in the nucleus and/or cytoplasm which were above any signal on the DapB slides. Cases where the ubiquitin C control was negative were considered to have inadequate RNA preservation and were not interpreted. All in situ hybridization slides were reviewed by three study pathologists (RFW, RDC, and JSL), and discrepancies were resolved by consensus review.

Reverse Transcriptase Polymerase Chain Reaction Detection of HPV E6/E7 mRNA

On the cases that were technical failures with HPV RNA in situ hybridization, reverse transcriptase polymerase chain reaction was performed using RNA extracted from unstained slides for the E6 and E7 proteins of the most common 13 high risk HPV types (16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66, and 68). Two 10 µm unstained sections were prepared and the corresponding H and E slides marked for tumor regions. The tumor containing regions of the unstained slides were macrodissected for RNA extraction, which was performed with Qiagen miRNeasy formalin-fixed, paraffin-embedded tissue kits and resulted in between 0.2 and 17 µg depending on tumor size. All oligo primers in the assays were purchased from Sigma-Aldrich (St. Louis, MO). The reverse transcription reaction was done with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The reverse transcriptase PCR reaction mixture contained 100 ng of RNA and was incubated at 25 °C for 20 min, 37 °C for 60 min, and then 85 °C for 5 min. Real-time PCR was then performed to quantify the cDNA product using Power SYBR Green PCR Master Mix (Applied Biosystems) and 500 nM HPV type-specific primers. Each HPV assay for E6 or E7 from 13 HPV types was individually performed in a separate well on a 384-well PCR plate. The PCR protocol was 95 °C for 10 min, and then 36 cycles of amplification (95 °C for 10 s, 58 °C for 15 s and 60 °C for 15 s). In addition, GAPDH and β-actin were included as expression reference controls for real-time PCR data normalization (normalized to the average expression of these two control genes). HPV quantitation was expressed as normalized log2-scale data based on detection-threshold PCR cycle number (Ct). A tumor sample was considered HPV negative if no fluorescent signal was detected within 33 PCR cycles (Ct > 33) [26].

Results

A total of 31 cases were identified, of which 22 (80 %) were biphasic with identifiable conventional SCC or squamous dysplasia (Fig. 1a). The remaining nine cases were composed of the spindle cell component only. The spindle cell component was highly variable between cases ranging from monomorphic spindle cell populations (Fig. 1b) to highly pleomorphic tumors with multi-nucleated cells, markedly pleomorphic nuclei, and numerous atypical mitotic figures, features reminiscent of the soft tissue tumor undifferentiated pleomorphic sarcoma (Fig. 1c). Some tumors showed a prominent myxoid stroma (Fig. 1d). A few cases also showed features

Fig. 1 Histologic features of spindle cell carcinoma. **a** Biphasic tumor with both conventional squamous cell and spindle cell components ($\times 200$ magnification) **b** Tumor with a monomorphic, spindle cell morphology ($\times 400$) **c** Markedly atypical tumor with morphology similar to pleomorphic undifferentiated sarcoma ($\times 400$)

d Tumor with prominent myxoid change resembling a myxoid sarcoma ($\times 400$) **e** Tumor mimicking granulation tissue ($\times 400$) **f** p63 positive monophasic spindle cell carcinoma ($\times 400$). (All images hematoxylin and eosin stained)

strongly mimicking granulation tissue with a loosely aggregated cells and an inflammatory background (Fig. 1e). None of the cases showed specific, clearly recognizable, sarcoma components such as chondrosarcoma or osteosarcoma. Cases that contained only a spindle cell component were included only if they were positive for at least one epithelial marker such as p63 (Fig. 1f) or pancytokeratin by immunohistochemistry in their routine clinical practice evaluation.

Demographic features of the patients are presented in Table 1. Most of the patients were Caucasian males with a history of smoking and drinking, and ages ranged from 39 to 82 years (average 64). These are comparable to conventional squamous cell carcinoma and other reports of

spindle cell carcinoma. We also examined the incidence of previous cancers and radiation exposure. In the 25 patients with adequate follow up information, nine (36 %) patients had a previous squamous cell carcinoma of the head and neck ranging from 2 to 13 years prior to their initial spindle cell carcinoma diagnosis, and six (24 %) had a history of radiation treatment to the head and neck.

Staging and clinical follow up data are presented in Table 2. The most common primary site was the oral cavity followed by the larynx, oropharynx, and hypopharynx. Of the patients in which staging information was available, most were stage IV. Lymph node metastases were present in six patients at initial diagnosis. The majority of patients were treated with primary surgery and

Table 1 Demographic features of the patient cohort

Demographics (n = 31)	
Age range (years)	39–82 (mean = 64.4)
Gender	
Male	25 (80.6 %)
Female	6 (19.4 %)
Male:female ratio	4:1
Race	
Caucasian	25 (80.6 %)
African-American	5 (16.1 %)
Other	1 (3.3 %)
Risk factors*	
Tobacco use (n = 24)	23 (95.8 %)
Alcohol use (n = 22)	21 (95.5 %)
History of radiation (n = 25)	6 (24.0 %)
History of prior SCC (n = 25)	9 (36.0 %)

SCC squamous cell carcinoma

* Adequate clinical documentation was available in 25 patients with the remaining 6 patients lost to follow up after primary diagnosis

eight had adjuvant radiation. The remaining six patients either died shortly after diagnosis or were lost to follow up. Average follow up was 106.6 months for surviving patients. At last follow up, only seven of 25 patients (28 %) were alive without disease. Local recurrence developed in 11 of 25 patients (44 %), regional (nodal) recurrence in seven patients (28 %), and distant metastasis in six patients (24 %).

HPV RNA in situ hybridization, RT-PCR, and p16 immunohistochemistry results are presented in Table 3. For the RNA in situ hybridization testing, two cases had to be excluded due to inadequate RNA preservation, and 9 cases had high background on the DapB negative control slides so were determined to be technical failures since we could not rule out low positive signals in the actual patient slides. To alternatively evaluate for HPV RNA, RT-PCR for high risk HPV E6 and E7 mRNA was performed on these 11 cases and was technically successful in all but one.

Only one of the 31 cases (3 %) was positive for p16 immunohistochemistry (Fig. 2a). That same case was also positive for HPV E6/E7 mRNA (Fig. 2b). It was a monophasic spindle cell carcinoma arising in the oral cavity on the left alveolar ridge without involvement of the oropharynx. One additional case was positive for HPV mRNA, but negative for p16. It was a biphasic spindle cell carcinoma of the larynx centered on the true cords and which had subglottic extension. In this case, the HPV RNA ISH staining was weakly but diffusely positive in both the conventional squamous cell carcinoma and spindle cell components. HPV mRNA testing in all 29 of the remaining

Table 2 Staging, treatment, and follow up information of the patient cohort

Location (n = 31)	
Oral cavity	12 (38.7 %)
Oropharynx	5 (16.1 %)
Larynx	11 (35.5 %)
Hypopharynx	3 (9.7 %)
Clinical stage at diagnosis (n = 31)	
I	7 (22.6 %)
II	6 (19.4 %)
III	1 (3.2 %)
IV	12 (38.7 %)
Not staged	5 (16.1 %)
Lymph node metastasis (n = 31)	
6 (19.4 %)	
Treatment (n = 23)*	
Surgery only	10 (43.5 %)
Surgery with radiation	8 (34.8 %)
Radiation only	3 (13 %)
Neoadjuvant chemotherapy	2 (8.7 %)
Disease progression (n = 25)*	
Local failure	11 (44 %)
Regional failure	7 (28 %)
Distant metastasis	6 (24 %)
Overall survival (n = 25)*	
Alive, disease free	7 (28 %)
Deceased with disease	13 (52 %)
Deceased, disease free	5 (20 %)

* Adequate clinical follow up information was available in 25 patients with the remaining 6 patients being lost to follow up after primary diagnosis. Treatment information was available on 23 patients

cases was negative, including all five oropharyngeal spindle cell carcinomas.

We examined clinical follow up information for the two HPV positive cases. For the first patient, who had a monophasic spindle cell carcinoma of the oral cavity which was positive for both HPV RNA and p16, the tumor was invading cortical bone at presentation and was clinically unresectable (T4b). Clinically there were no nodal metastases (N0). He received neoadjuvant chemotherapy, but died of progressive disease at 22 months. For the second patient, who had a biphasic spindle cell carcinoma of the glottis which was positive for HPV RNA but negative for p16, the tumor was surgically resected by a total laryngectomy with bilateral neck dissections. The tumor invaded through the thyroid cartilage (T4a) and had bilateral cervical nodal metastases (N2b). He declined postoperative radiation therapy, however, and went on to develop regional recurrence in cervical lymph nodes after 5 months. He died of disease 9 months after diagnosis.

Table 3 HPV testing results by anatomic subsite

Anatomic site	p16 Immunohistochemistry	HPV RNA ISH (n = 20)	HPV RT-PCR (n = 10)	HPV (by either assay)
Oral cavity	1/12 (8 %)*	1/9 (11 %)	0/3 (0 %)	1/12 (8 %)
Oropharynx	0/5 (0 %)	0/4 (0 %)	0/1 (0 %)	0/5 (0 %)
Larynx/hypopharynx	0/14 (0 %)	1/7 (14 %)	0/6 (0 %)	1/13 (8 %)
Totals	1/31 (3 %)	2/20 (10 %)	0/10 (0 %)	0/30 (0 %)**

ISH in situ hybridization, RT-PCR reverse transcriptase polymerase chain reaction

* This case was positive for both p16 immunohistochemistry and HPV RNA ISH

** One case lacked adequate RNA preservation for HPV specific testing

Fig. 2 p16 immunohistochemistry and RNA in situ hybridization. **a** p16 positive tumor with diffuse and strong cytoplasmic and nuclear staining ($\times 600$ magnification) **b** Same tumor with positive RNA in situ hybridization with brown, granular, cytoplasmic staining ($\times 600$)

Discussion

Spindle cell carcinoma is an uncommon variant of head and neck squamous cell carcinoma that represents divergent (mesenchymal) differentiation from an epithelial neoplasm. Our study population conforms to the previously established demographics for these tumors, with a predilection for men in their 5th and 6th decades and an almost uniformly positive history of tobacco and alcohol use. Prior head and neck cancer is common and prior radiation therapy for other tumors/diseases is also quite common in our study population and in the literature [27]. Local and regional recurrences and distant metastases occur with regularity, comparable to, or slightly more commonly than for, conventional squamous cell carcinoma [1, 4, 7].

The role of HPV is well established in oropharyngeal squamous cell carcinoma [28] and many squamous cell carcinoma variants in the oropharynx and other head and neck subsites [21]. Through the direct inhibition of the tumor suppressor genes p53 and Rb by the viral proteins E6 and E7 there is dysregulation of the cell cycle and loss of normal apoptotic mechanisms and overexpression of p16. It is also clear from both clinical trials and epidemiologic data that the presence of transcriptionally-active HPV has deep clinical and prognostic implications, strongly

correlating with improved survival in oropharyngeal squamous cell carcinoma. Studies have also established the presence of transcriptionally-active HPV in variable subsets of specific squamous cell carcinoma histologic variants when they arise in the oropharynx or sinonasal region. This includes adenosquamous carcinoma, high grade neuroendocrine/small cell carcinoma, basaloid squamous cell carcinoma, undifferentiated carcinoma, and papillary squamous cell carcinoma [22–24, 29–31]. Where prognostic data has been compiled, the presence of HPV appears to be correlated with better outcomes in several of these variants, perhaps with the exception of high grade neuroendocrine/small cell carcinomas, in which the morphology appears to “trump” the presence of HPV in dictating outcomes.

To our knowledge, there are no published studies evaluating head and neck spindle cell carcinomas for HPV. Given the other major risk factors for this tumor such as smoking, drinking, and previous radiation exposure and given the lack of any predilection of spindle cell carcinoma for the oropharynx, one would not necessarily suspect these tumors to harbor transcriptionally-active HPV. However, one published abstract, by Kryvenko et al. [25], reported the evaluation of 14 cases of head and neck spindle cell carcinoma for p16 expression and high risk HPV by

chromogenic DNA in situ hybridization. They reported 5 cases (35.7 %) to be p16 positive and 12 cases (85.7 %) to be positive for HPV.

In the current study, utilizing a now well-established and ultrasensitive RNA in situ hybridization assay which correlates strongly with other HPV tests and with clinical outcomes in oropharyngeal squamous cell carcinoma, we find that the majority of spindle cell carcinoma cases lack transcriptionally-active HPV. Only one patient had detectable HPV E6/E7 mRNA and p16 expression. In a second patient who was positive only for HPV mRNA, it appears to be represent biologically active virus in the absence of p16 overexpression. Perhaps there were separate genetic alterations leading to the inactivation of p16 even in the presence of active virus. Although eleven of the cases were technical failures by RNA in situ hybridization due to low level obscuring signals on the DapB negative controls or lack of adequate ubiquitin C positive internal control staining, RT-PCR was performed for HPV E6 and E7 transcripts as a secondary test for transcriptionally-active HPV. It was negative in all 10 cases (one had inadequate RNA preservation). Further, all five of the oropharyngeal primaries were technically evaluable by RNA ISH and none were HPV positive. Clinical follow up data for the two HPV mRNA positive patients showed both to have very locally and regionally advanced tumors, and both patients died of disease within 2 years. These findings suggest that even spindle cell carcinoma cases with transcriptionally active HPV are not associated with a more favorable outcome. However, with only two HPV positive patients, neither of whom could have aggressive medical treatment, no definitive conclusions regarding prognosis can be drawn.

In summary, spindle cell carcinomas, with rare exception, lack transcriptionally-active HPV, even when arising in the oropharynx. Furthermore, the rare presence of HPV in these tumors does not appear to be associated with improved prognosis. These findings argue that smoking, drinking, and radiation exposure are the critical risk factors for the development and progression of such tumors and that HPV vaccines and targeted therapies will not be useful for spindle cell carcinoma.

Acknowledgments The authors would like to acknowledge Jianping Li, BS and Neha Dahiya, MD, MBA of the Anatomic and Molecular Pathology Core Laboratory for their expert assistance with the p16 immunohistochemistry.

Conflict of interest The RNA in situ hybridization assays used in this study were funded by and performed by the co-authors from Advanced Cell Diagnostics, Inc., Hayward, CA. These three co-authors (HW, YL, XM) all have stock in this company and stand to profit by any use of this testing through their company. The remaining co-authors have received nothing from the company and have no financial or other conflicts of interest to disclose.

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