Transcriptionally-Active Human Papillomavirus is Consistently Retained in the Distant Metastases of Primary Oropharyngeal Carcinomas

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Received: 15 October 2013 / Accepted: 14 November 2013 / Published online: 23 November 2013
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Abstract High-risk human papillomavirus (HPV) is both causative and prognostic in the majority of oropharyngeal squamous cell carcinomas (OPSCCs). The aim of this study was to evaluate for transcriptionally-active HPV in matched primary OPSCCs and their distant metastases given the implications of HPV status for diagnosis and treatment. Twenty matched pairs of primary OPSCC and their distant metastases were retrieved from departmental files. Two study pathologists reviewed all cases to confirm the diagnoses and to evaluate histologic features. Real-time PCR (RT-PCR) for detection of E6/E7 mRNA for all major high-risk HPV types and p16 immunohistochemistry were performed. Distant metastases were to lung (70 %), bone (20 %), non-regional lymph nodes (5 %) and pericardium (5 %). Histologically, 15 primary tumors were nonkeratinizing, 3 nonkeratinizing with maturation, one basaloid, and one keratinizing. Seventeen (85 %) of the metastases had the same histologic type as the primary tumor. All 20

matched pairs were concordant for HPV status by RT-PCR and for p16 expression with 19 of 20 cases positive for high risk HPV and one negative. HPV types were concordant in all cases. These findings show that the distant metastases from HPV-related primary OPSCCs uniformly retain transcriptionally-active HPV and p16 overexpression. They also retain similar morphology. This argues that HPV status can be utilized to differentiate metastatic OPSCC from separate, new, primary squamous cell carcinomas in other organs, and that therapies specifically targeting HPV or virus-related proteins in patients with distant metastases can be utilized.

Keywords Squamous cell carcinoma · Oropharynx · Human papillomavirus · Distant metastasis · p16 · Nonkeratinizing

Introduction

High-risk human papillomavirus (HPV) infection is causative in the majority of oropharyngeal squamous cell carcinomas (OPSCCs) in North America [1, 2] and much of the rest of the world. HPV-related OPSCCs are clearly distinct from their HPV negative counterparts, in terms of epidemiological, molecular, and histopathological features and have improved treatment responses and patient outcomes despite the high rate of regional lymph node metastases [3, 4, 5, 6].

Distant metastases in OPSCCs are relatively uncommon (10 % or less of patients compared to 12–15 % for typical head and neck squamous cell carcinomas), particularly in surgically treated patients [3, 7–10]. However, they still represent the major form of disease recurrence and lead to poor outcome in such patients. Interestingly, the reported

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overall distant metastasis rate has been shown to be similar between HPV positive and negative patients [3, 8, 10]. A few recent studies, though, have suggested clinically distinct differences between HPV positive and negative tumors for the course and patterns of disease dissemination [8, 10, 11], including a longer interval between treatment of the primary tumor and development of metastases and differences in organs involved and in the number of metastases. The lung is the most common site for the distant metastases from HPV-related OPSCCs and also the most common site for development of second primary squamous cell carcinomas in head and neck cancer patients [12, 8, 10]. The distinction between the two is crucial with major impact on patient management and prognosis.

HPV has traditionally been detected by DNA-based PCR [13]. However, this only evaluates the simple presence of the virus. It has become very clear that it is not just the presence of HPV, but its presence in transcriptionally-active form, that is critical for clinical relevance [14, 15]. In oropharyngeal squamous cell carcinoma, HPV can be present in either integrated and/or episomal form. The general thinking is that integration is necessary for carcinogenesis because it disrupts HPV regulation leading to constitutive overexpression of early genes necessary for disruption of host cell pathways. In cervical [16] and in oropharyngeal squamous cell carcinoma, studies have shown as many as 30 % or more of cases to lack evidence of integration [17, 18]. Although much research has focused on the oncogenic pathways that are important in primary oropharyngeal squamous cell carcinoma, minimal information exists about HPV in distant metastatic lesions, and no studies have thoroughly evaluated the distant metastases of these tumors to see if the HPV is consistently retained in them. The aim of this study was to evaluate for histopathological characteristics and for transcriptionally-active HPV in primary OPSCCs and their matched distant metastases.

Materials and Methods

After Human Research Protection Office (institutional research ethics board) approval, a retrospective review was conducted on all primary oropharyngeal squamous cell carcinoma patients in a large database ($n = 270$) at Washington University School of Medicine in whom distant metastases developed [14]. This “parent” database was retrospectively accrued and included patients treated by primary surgery or primary chemoradiotherapy. For this study, inclusion criteria for patients were new oropharyngeal primary SCC with distant metastasis(es), available tissue blocks from both the primary tumor and from a focus of distant metastasis, and available clinical follow up

information from the medical record. The most common reasons for exclusion were lack of a surgical (tissue) specimen from the distant metastasis (for example, patients either underwent fine needle aspiration or had no diagnostic biopsy of their distant metastasis performed at all—rather were just diagnosed clinically) and lack of availability in our files of either the primary or distant metastasis specimens for testing.

The slides were retrieved from surgical pathology files and reviewed by two study pathologists (MM, JSL) to confirm the diagnosis of squamous cell carcinoma and to evaluate the histologic features. Tumors were typed with a previously described, established system [19, 20] as non-keratinizing, nonkeratinizing with maturation, or keratinizing type squamous cell carcinoma. The specific variant basaloid squamous cell carcinoma was diagnosed based on established World Health Organization morphologic features [21]. Discrepant cases were resolved by consensus review. The medical records of the patients were reviewed for clinical features, imaging results, and treatment. The presence of metastasis (rather than development of a new primary squamous cell carcinoma) in distant sites was based on the clinical impression of the treating oncologist or radiation oncologist.

p16 Immunohistochemistry

Immunohistochemistry was performed using a Ventana Benchmark automated stainer using p16 antibody (MTM Laboratories; clone E6H4; monoclonal; 1:1 dilution). Controls were a known HPV (and p16) positive oropharyngeal carcinoma and normal tonsil. All cases were reviewed independently, without knowledge of the HPV results, by two (MM, JSL) study pathologists. The staining pattern for p16 was classified as follows (with tumor cells being considered positive when showing nuclear and cytoplasmic staining, regardless of intensity): 0 = negative; 1+ = 1–25 % of tumor cells positive; 2+ = 26–50 %; 3+ = 51–75 %; 4+ = ≥ 76 %. Results were dichotomized as 4+ being positive and all others negative. Discrepant results were resolved by consensus review.

Reverse-Transcriptase Real Time PCR (RT-PCR)

RT-PCR was performed using RNA extracted from two 10 μm unstained slides which were selected for tumor containing areas from the corresponding H&E slides [22]. Testing was performed for E6 and E7 mRNA transcripts of the most common 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66, and 68). Extraction was with Qiagen miRNeasy formalin-fixed, paraffin-embedded tissue kits (QIAGEN Sciences Inc., Gaithersburg, MD)

and resulted in between 0.5 and 5 µg of RNA, depending on tumor size. All oligo primers in the assays were purchased from Sigma-Aldrich (St Louis, MO, USA). The reverse-transcription reaction was done with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The RT-PCR reaction mixture contained 150 ng of RNA, and was incubated at 25 °C for 20 min, 37 °C for 60 min, and then 85 °C for 5 min. RT-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). GAPDH and β-actin were included as expression reference controls for RT-PCR data normalization (normalized to the average expression of these two control genes). Quantitation was expressed as normalized log₂-scaled data compared with the detection threshold PCR cycle number.

Results

A total of 29 patients were identified from the larger database who had a primary oropharyngeal squamous cell carcinoma and went on to develop a distant metastasis. Of these, we were able to obtain matched primary tumor and distant metastasis tissue blocks for 20 patients. Cases were from 1996 to 2012.

Distant metastases occurred in the lung (14/20, 70 %), bone (4/20, 20 %), non-regional lymph nodes (1/20, 5 %) and pericardium (1/20, 5 %) (Table 1). At presentation, none of the patients showed evidence of multiple organ involvement. Distant metastases occurred within a range of 0–40 months after diagnosis of the primary tumor, with a mean and median of 15.6 and 16.5 months, respectively. Of note, 40 % of the HPV positive distant metastases developed after 2 years.

The results of morphologic examination and HPV and p16 testing are summarized in Table 2. Histologically, 15 primary tumors were nonkeratinizing, three were nonkeratinizing with maturation, one basaloid, and one keratinizing squamous cell carcinoma (Fig. 1). Seventeen (85 %) of the metastases had the same histologic type as the primary tumor. p16 immunohistochemistry was extensively

Table 1 Clinical features of the patients including specific details of the distant metastasis(es)

Case#	Stage at presentation ^a	Treatment	Interval to distant metastasis(es) (mo)	Location of distant metastasis(es)	Number of foci of distant metastasis
1	I (T1/N0/M0)	Chemo/rad	31	Lung	1
2	IVA (T3/N2/M0)	Surgery + chemo/rad	23	Lung	1
3	IVA (T2/N2b/M0)	Chemo/rad	23	Lung	2
4	IVB (T2/N3/M0)	Chemo/rad	7	Bone, Femur	1
5	IVA (T2/N2c/M0)	Chemo/rad	13	Lymph nodes 4R, 3, 7	3
6	III (T3/N2/M0)	Surgery +chemo/rad	23	Lung	1
7	IVA (T2/N2/M0)	Surgery +chemo/rad	24	Bilateral lung	2
8	IVA (T1/N2/M0)	Surgery +chemo/rad	16	Lung	1
9	III (T1/N1/M0)	Surgery +chemo/rad	21	Lung	1
10	IVA (T2/N2/M0)	Surgery +chemo/rad	30	Lung	1
11	IVA (T4a/N2a/M0)	Chemo/rad	11	Lung	1
12	IVA (T2/N2b/M0)	Chemo/rad	17	Lung	1
13	IVC (M1)	Chemo/rad	0	Bone, Femur	1
14	IVB (T1/N3/M0)	Surgery, chemo/rad	9	Lung	1
15	IVB (T4b/N2c/M0)	Chemo/rad	10	Lung	1
16	IVC (M1)	Chemo/rad	0	Pericardial fluid	1
17	IVC (M1)	Chemo/rad	0	Bone	1
18	IVA (T2/N2/M0)	Surgery + chemo/rad	9	Lung	1
19	III (T2/N2/M0)	Chemo/rad	15	Bilateral lung	2
20	IVB (T1/N3/M0)	Surgery + chemo/rad	40	Bone	1

chemo chemotherapy; rad radiation therapy

^a Patients treated by primary chemotherapy and radiation were staged based on clinical features

Table 2 Morphology and p16 and human papillomavirus status by individual patient

Case	Nonkeratinizing		Nonkeratinizing with maturation		Basaloid		Keratinizing		p16 IHC		High risk HPV RT-PCR	
	Prim	Met	Prim	Met	Prim	Met	Prim	Met	Prim	Met	Prim	Met
1	+					+			+	+	16	16
2	+	+							+	+	16	16
3	+	+							+	+	16	16
4	+	+							+	+	16	16
5	+	+							+	+	16	16
6	+	+							+	+	16	16
7	+	+							+	+	16	16
8	+	+							+	+	16	16
9	+	+							+	+	16	16
10	+	+							+	+	16	16
11	+	+							+	+	18	18
12	+	+							+	+	35	35
13	+	+							+	+	16	16
14	+			+					+	+	16	16
15	+			+					+	+	16	16
16		+	+						+	+	16	16
17			+	+					+	+	16	16
18			+	+					+	+	16	16
19					+	+			+	+	16	16
20							+	+	–	–	–	–

IHC immunohistochemistry; *prim* primary tumor; *met* distant metastasis

(4+) positive in 19 of the primary tumors and negative (0) in one. The matched distant metastases had identical results. The single case that was negative for p16 was a keratinizing type squamous cell carcinoma.

All 20 matched pairs were concordant for HPV mRNA status by RT-PCR with 19 of 20 positive for high risk HPV. Three different types of HPV were identified: HPV-16 in 17 pairs and HPV-18 and HPV-35 in a single pair each (Table 2). HPV transcriptional activity was assessed relative to control genes for the 19 positive cases. There was no significant difference between primary tumors (range 2.0–12.1 units; mean 5.9) and their distant metastases (range 1.7–8.6 units; mean 5.8).

Discussion

Only a few recent studies have evaluated the distant metastases in HPV-related/p16 positive OPSCCs. Huang et al. [10] described 36 patients with oropharyngeal SCC (24 p16 positive and 12 p16 negative) from a cohort of 318 patients treated with definitive radiation therapy with or without concurrent chemotherapy. They reported an atypical clinical behavior for the p16 positive patients, including distant metastases to multiple organs and to

unusual sites such as the brain and skin, and also reported a longer interval to distant metastasis in p16 positive patients versus those who were p16 negative. Later, the same group of researchers [8] studied the natural course of distant metastases in 624 patients over 10 years treated with definitive radiation with or without chemotherapy for oropharyngeal squamous cell carcinoma. There were 54 p16 positive and 25 p16 negative distant metastasis patients. Again, they found that even though the overall distant metastasis rates are similar, there was a longer interval to their development in the p16 positive patients. Additionally they reported a differing pattern for the metastases which they called “disseminating”, defined as presence of them in multiple (>2) organs. The most common organ involved was still the lung. They did not, however, describe the histologic features of the metastases nor did they evaluate the metastases to see if transcriptionally-active HPV was retained.

Recently Müller et al. [11] described the clinical, histopathologic, and immunophenotypic features of four cases of HPV-related oropharyngeal squamous cell carcinoma and their distant metastases. They tested tissue specimens from both the primary tumors and matched distant metastases in all four cases and showed all to be strongly and diffusely p16 positive. In the one case where they tested

Fig. 1 Histologic features of oropharyngeal squamous cell carcinoma and distant metastases. Primary tumor (A) and its corresponding distant metastasis (B) with both foci showing the nonkeratinizing morphology that is typical of HPV-related OPSCCs with haphazardly

arranged lobules of blue tumor with cells with scant cytoplasm. The primary tumor (C) and lung metastasis (D) were strongly and diffusely positive for p16 immunohistochemistry. (All panels 10X magnification; HPV = human papillomavirus)

both the primary and distant metastasis for high risk HPV (by DNA in situ hybridization), it was positive at both sites. They also reported that the tumor morphology was retained in the distant metastases.

This examination of the current literature shows that there has been no extensive examination of the tissue specimens from patients with distant metastatic oropharyngeal squamous cell carcinoma to see if the morphology, p16, and HPV status are retained and, if so, if the HPV has the same level of transcriptional activity. It seems that the medical community has largely just assumed that the HPV “drives” tumor growth wherever the squamous cell carcinomas spread, probably because of the assumption that HPV is integrated into the host genome. In cervical cancer, integration rates are high (on the order of 70 %) [23, 24]. There are less studies in oropharyngeal squamous cell carcinoma and results have been similar, but slightly more variable [17,

25, 18]. Why can the HPV not be shed or lost as a tumor progresses genetically and gains the ability to metastasize? The knowledge that HPV is consistently retained and in similarly active form is critical for future therapies that target not only the virus itself in patients with distant disease, but also for therapies that target the molecular pathways driven by the virus. Data from the current study, finding consistent retention of morphology, p16, and HPV status, supports the notion that active high risk HPV continues to drive tumor growth and spread in all cases. In this study, 19 of the 20 (95 %) cases were positive for high-risk HPV. This rate is slightly higher than has been reported in almost every large study on oropharyngeal squamous cell carcinoma and may partly be due to several factors such as the low number of cases in our study, to idiosyncrasies in what cases had material available from the pathology files for study, and/or to the fact that most patients with

HPV-negative oropharyngeal SCC have local and/or regional recurrence when they develop distant metastasis. This latter feature is different than for HPV-related oropharyngeal SCC, where most patients with distant metastases have it as isolated recurrence. Given the prolonged survival they experience, there probably were more lung wedge biopsies/resections so more patients with tissue from their distant metastases available for study [8, 10].

This knowledge of HPV retention is also critical for whether HPV status can be used to differentiate distant metastases from new primary tumors. Although complex molecular methods such as gene mutation analysis and/or microsatellite alterations [26, 27] can be used for this purpose, their implementation in routine clinical practice has been limited due to cost, technical availability, and the inherent instability of a tumor's genetic profile over time [28]. Our data supports the use of, by contrast, relatively simple HPV testing for differentiation of second primary tumors from distant metastases of OPSCCs. Most oropharyngeal squamous cell carcinoma distant metastases are to the lung so that one must discern metastasis from a new primary lung squamous cell carcinoma. There are some important clinical clues such as uni- versus multifocality and shape of tumor (speculated versus lobulated/smooth bordered), but these are often not definitive. Morphology, p16 status, and HPV-specific testing are the pathological features that can help. The question then becomes “what is the rate of p16 and HPV positivity among primary lung squamous cell carcinomas?” The reported frequencies of HPV DNA in lung cancers of all types vary widely depending on the geographical location, ranging from 0 to 78.3 % (mean 24.3 %), with more prevalence in Asia and less in North America, South America, and Europe [29, 30]. These studies show relatively high rates of HPV DNA, but we know from other anatomic subsites that HPV DNA can be found in many tumors without any activity or clinical relevance. High risk HPV transcriptional activity is best assessed by the detection of E6/E7 mRNA and p16 overexpression. Yanagawa et al. [31] studied 336 “clinically” primary non-small cell lung carcinomas from North American patients by staining of tissue microarrays for p16 and for high risk HPV by in situ hybridization and reported an overall HPV frequency of 1.5 % (n = 5). These five cases were confirmed as HPV positive by DNA-based PCR. Interestingly, all five HPV positive lung tumors were type 16, were squamous cell carcinomas, and all patients had history of HPV-related squamous cell carcinoma at another anatomic site. Therefore, the authors felt strongly that these tumors were metastases to the lung rather than new primaries. In addition, they performed p16 immunohistochemistry on all cases with a cutoff of 10 % or more expression as positive. All 5 HPV positive tumors were positive, but overall, 109 (32.4 %) tumors were positive

including 24 of 132 (18.2 %) of the squamous cell carcinomas. These data suggest that transcriptionally-active high risk HPV is rare to non-existent in primary lung carcinomas but that p16 expression is relatively common.

A few studies have specifically tried to use HPV status to discern primary lung squamous cell carcinomas from metastases [32]. One study, in particular, by Bishop et al. [33] using p16 immunohistochemistry and HPV DNA in situ hybridization showed no HPV in 66 primary lung squamous cell carcinomas, but p16 overexpression (using a stringent cutoff of 70 %) in 21 % of tumors. They found that 15 of 28 (54 %) of patients with primary OPSCCs had HPV positive and p16 overexpressing lung tumors, arguing strongly that these lung tumors were metastases from their oropharyngeal primaries. Collectively, the literature argues that, although HPV DNA can commonly be found in non-small cell lung carcinomas in North American patients, transcriptionally-active high risk HPV is rare. p16 overexpression is a sensitive, although nonspecific, surrogate marker for its presence. As such, we recommend caution when utilizing HPV testing to distinguish primary lung squamous cell carcinomas from metastases in patients with a history of HPV-related oropharyngeal squamous cell carcinoma. Our recommendation would be to 1) compare morphologic features to see if the lung tumor is nonkeratinizing and of similar histology to the oropharyngeal primary 2) test for p16 immunohistochemistry using a high cutoff (>70 %) for positivity and, only if positive, 3) perform HPV specific testing by whatever test is available to the pathologist clinically, be it HPV DNA in situ hybridization or PCR. The finding of a p16 overexpressing, HPV DNA or RNA positive lung tumor is then very strong evidence that it represents a metastasis.

In summary, the distant metastases from HPV-related primary OPSCCs appear to uniformly retain HPV and p16 status and with the same rate of transcriptional activity for HPV E6 and E7 oncoproteins. They also largely retain the same histologic features. These findings have major treatment implications for any future agents that would specifically target HPV proteins or the molecules altered by transcriptionally-active HPV in such patients because they show that these molecules, and the biological pathways critical for HPV-related tumor growth and progression, remain intact wherever the tumors spread. Finally, the finding of late metastases (>2 years from primary diagnosis) among HPV-related oropharyngeal squamous cell carcinoma patients suggests that extended patient follow-up is probably necessary in these patients.

Acknowledgments The authors would like to acknowledge Autumn Watson B.A. and Neha Dahiya, M.D., M.B.A. of the Anatomic and Molecular Pathology Core Laboratory for their expert assistance with the p16 immunohistochemistry.

Conflict of interest The authors have no financial or other conflicts of interest to disclose.

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