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Preliminary Results from Retrospective Correlation of Circulating Tumor DNA (ct-DNA) with Imaging for HPV-positive Oropharyngeal Squamous Cell Carcinoma

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ABSTRACT

The role of molecular markers is increasingly being recognized for head and neck tumors ranging from benign lesions like paragangliomas to malignancies like squamous cell carcinomas (SCCa). Multiple studies have recently validated blood tests for circulating tumor tissue modified viral- human papillomavirus DNA (HPV ct-DNA) (*NavDx, Naveris Laboratories*) for posttreatment surveillance of HPV-driven oropharyngeal SCCa. This technology quantifies fragments of circulating DNA that are shed into the blood stream with very high (>95%) positive and negative predictive values and are also highly sensitive in distinguishing tumor HPV-DNA from a non-cancerous source. This study has a cohort of 34 patients with HPV-driven oropharyngeal SCCa, having at least three sequential imaging studies and ct-DNA values. The study showed a strong positive correlation between the imaging findings and ct-DNA level in recurrent HPV positive oropharyngeal SCCa. Findings also include 100% negative predictive value of HPV ct-DNA tests to rule out tumor recurrence. At our institution, we are now routinely performing the ct-DNA assay for surveillance of treated HPV- oropharyngeal SCCa. Correlation between clinical, radiological, and biomarker findings are now part of routine discussions during the multidisciplinary tumor boards.

ABBREVIATIONS: ct-DNA=circulating tumor deoxyribonucleic acid; HPV=Human Papilloma virus;OPC=Oropharyngeal SCCa=Squamous cell carcinomas; PCR= Polymerase chain reaction

From the Departments of Radiology (A.A, A.A.B, G.B, J.M, P.R) and Department of Otolaryngology (S.P), Mayo Clinic, Jacksonville, Florida

Conflict of interests: None for all authors. Please address correspondence to Amit Agarwal, MD, Departments of Radiology, Mayo Clinic, 4500 San Pablo Rd, Jacksonville, FL 32224; e-mail: Agarwal.amit@mayo.edu

INTRODUCTION

Over the last decade, there has been a gradual shift in the etiology of head and neck squamous cell carcinomas (SCCa) with a decline in cancers secondary to environmental exposures such as tobacco and alcohol and concomitant increase in cancers associated with human papillomavirus (HPV) infection. The incidence of HPV-associated SCCa has risen exponentially over the last decade, especially in high-income countries, now accounting for 71% and 51.8% of all oropharyngeal SCCa in the USA and UK, respectively.[1] Although, the prognosis of this subset of patients is significantly better than the non-HPV counterpart, the relapse/recurrence rate is still high, with around 10-25% of patients presenting with locoregional or distant metastasis in the surveillance period.[2] The post-treatment surveillance for HPV driven oropharyngeal SCCa relies on clinical examination, endoscopy and serial imaging studies as outlined by the National Comprehensive Cancer Network (NCCN) guidelines (version 3.2024). This includes guidelines for short-term (<6 months) and for long-term (6 months -5 years) surveillance. Standard imaging surveillance includes contrast-enhanced CT neck and PET-CT 3-6 months post-treatment.[4] As per the 2024 NCCN guidelines, the optimal timing for PET-CT exam is 3-6

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months with significant false-positive rates in studies done before 12 weeks. Negative PET-CT at 3-6 months predicts improved overall survival at 2 years . Subsequent follow-up imaging surveillance is however variable across institutions with poor consensus.[4] Imaging studies are limited in their accuracy demarcating post-treatment changes versus tumor recurrence, frequently leading to invasive procedures and biopsy for confirmation. Moreover, early and clinically occult recurrent tumor may also be missed on imaging. Circulating tumor DNA (ct-DNA) provides a quantitative measure of the tumor-modified HPV DNA which is different from non-cancerous HPV seen in the general population. ct-DNA provides an ultrasensitive quantitative measure of post-treatment tumor status, with studies showing 94-100% sensitivity and negative predictive value (NPV) and approximately 95% positive predictive value (PPV). [3,5,6] Tumor-modified HPV ct-DNA is a unique biomarker of HPV-associated malignancies with quantifiable levels in the blood and saliva of patients. The prevalence of various HPV subtypes differs by geographic region with HPV16 being the most common subtype in HPV driven oropharyngeal SCCa in the United States.[1] Routine testing of ct-DNA offers a more complete picture of the tumor status than obtained by imaging studies alone. [6,7] In a three-year longitudinal study by *Chera* et al, 100% of post-treated patients (n=115) with negative ct-DNA had no cancer recurrence whereas 94% of patients with two positive (rising) ct-DNA values had recurrence.[8] Combination of imaging findings and the biomarker assay can together provide an exceptionally high level of sensitivity and can complement each other in ambiguous cases.

MATERIAL & METHOD

After Institutional Review Board (IRB) approval, 38 cases of p16+ (HPV driven) oropharyngeal SCCa, were retrospectively identified via PACS search engine (Illuminate, PatientView), from June 2022 to March 2023. The study included patients with definite p16 positivity, presence of at least three sequential imaging studies and ct-DNA values, including pre-treatment ct-DNA values and imaging. Patients with equivocal p16 status but treated as HPV-induced SCCa were excluded from the study. The study also excluded patients with no pre-treatment ct-DNA values. A total of 34 cases met inclusion criteria of longitudinal imaging and ct-DNA tests, including baseline, pre- and post-treatment, sequential imaging studies and ct-DNA values (at least 3). There were two patients with equivocal HPV positivity on p16 stains and were excluded from the study. Two patients with no pre-treatment ct-DNA values were also excluded. **Supplemental Table 1** provides the details of patients age, gender, location of primary tumor, initial stage, HPV subtype along with treatment details.

Technical specifications:

Circulating-tumor DNA (ct-DNA) test isolates circulating free DNA from plasma using droplet digital PCR using 17 biomarkers. Millions of single HPV-DNA fragments containing droplets are allocated to 16 size pool clusters and an algorithm generates a tumor-tissue modified viral (TTMV) HPV DNA prognostic risk score for cancer recurrence. These tests distinguish (TTMV) HPV DNA from other non-cancerous sources of DNA, with results available within seven days. The test can be used pretreatment, during treatment, and post-treatment. The pre-treatment score provides an important baseline score depending on tumor burden and identifies the HPV strain. The effectiveness of treatment response is evaluated by tests during treatment, and finally, post-treatment tests is used for long-term surveillance. The result chart is updated after every test providing a graph along with absolute values (**Fig 1**). TTMV-HPV-16 DNA score of <2 is negative, 2-3 is indeterminate and >3 is positive.

RESULTS

Concordant negative ct-DNA and imaging (*most common*): Twenty-two (22) out of 34 patients (64.7%) had negative ct-DNA values obtained over a course of 6-9 months (**Fig. 1**). Clinical examination including endoscopy was performed in all these patients and were negative in all but one patient where deep ulceration and edema resulted in suboptimal evaluation of the oropharynx.

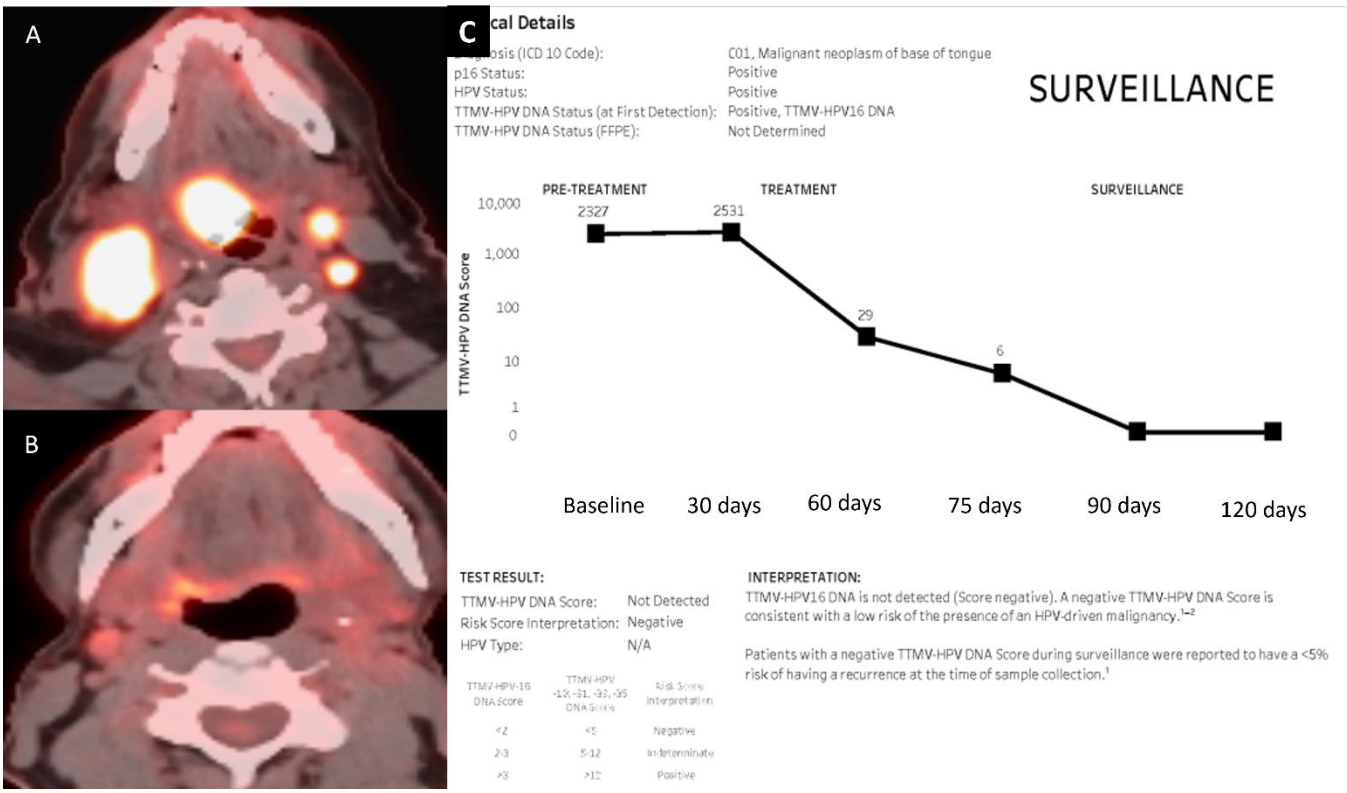

FIG 1. Right base of tongue SCCa (p16 +) with bilateral nodal metastasis with high uptake on initial PET-CT (A) and near-complete treatment response on follow up study (B). The ct-DNA (NavDx) results (C) showing concordant values with pre-treatment value of 2357 and negative (< 2) results on surveillance with quantitative value of 0. Mild increase in ct-DNA values noted on the initial treatment test (2531) secondary to tumor necrosis. SCCa- squamous cell carcinoma.

Discordant ct-DNA and imaging (*uncommon*): Eight (8) out of 34 patients (23.5%) had discordant results. Seven (7) out of these eight had patients had negative ct-DNA results and positive or indeterminate findings on imaging, whereas one patient had positive ct-DNA with negative imaging. In the former group (negative ct-DNA and positive imaging) four patients had increased oropharyngeal uptake on PET-CT which was interpreted as "concerning for tumor recurrence" (**Fig. 2**). Two patients had increased nodal uptake and one patient had increased uptake of pulmonary nodules on PET-CT exams. These (7 of 8) patients with discordant results were followed up with a combination of clinical/endoscopic exam, ct-DNA and PET-CT with no findings of recurrence on longitudinal follow-up over the next 4-6 months. Tissue biopsy was deemed unnecessary given the low suspicion for recurrence based on the negative clinical and endoscopic exams and continued negative ct-DNA results. One of eight had pathology-proven tumor recurrence where imaging was negative, interpreted as "post-treatment changes", with however, positive ct-DNA results. The decision to biopsy in this patient was made based upon the increasing ct-DNA values

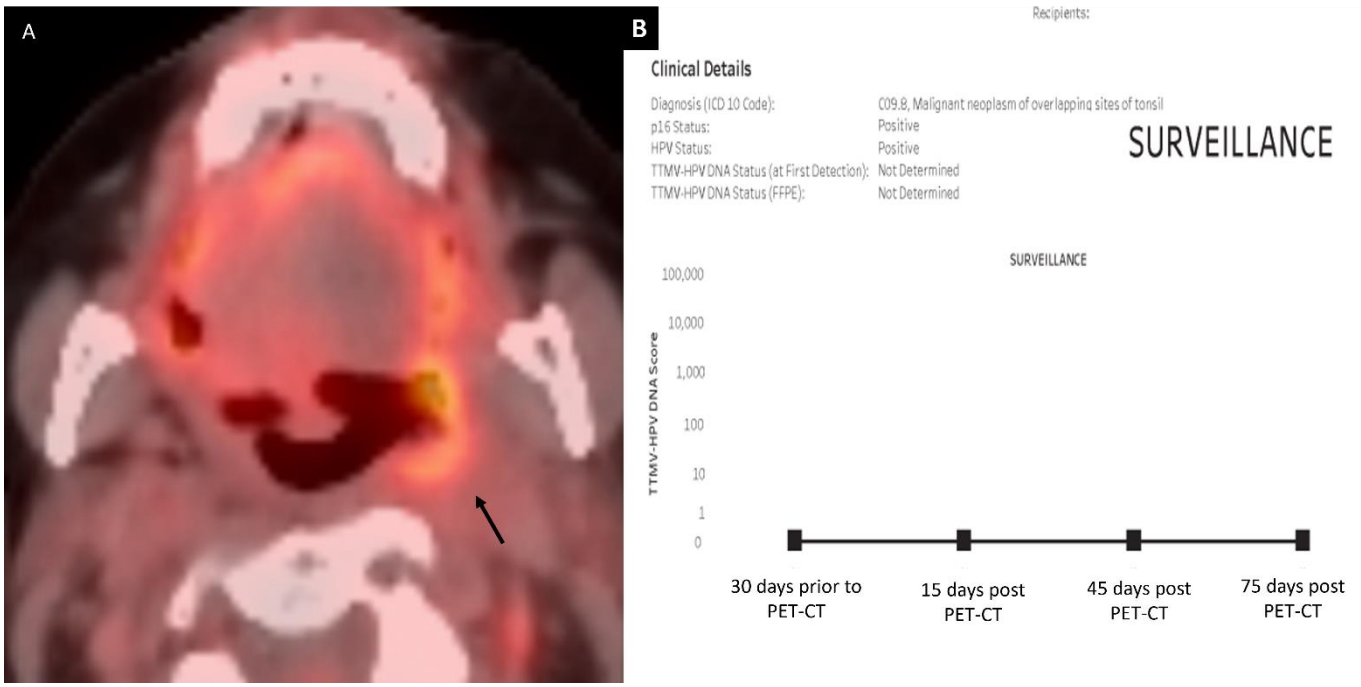

FIG 2. Discordance between imaging and ct-DNA results. Surveillance PET-CT (A) in a patient with treated p16+ oropharyngeal SCCa shows a nodular focus with increase FDG uptake in the left tonsillar fossa (black arrows) concerning for tumor recurrence. Multiple surveillance ct-DNA tests (B) during the same period however showed negative values and the consensus during the multidisciplinary tumor board was against any intervention. Follow-up PET CT (not shown) revealed complete resolution of uptake with findings consistent with post-treatment changes.

Concordant positive ct-DNA and imaging (*least common*): Four out of the thirty-four patients (11.7%) had tumor recurrence detected on PET-CT with positive ct-DNA with temporal increase in values. Two of these were localized and treated with curative intent (**Fig.3**) with negative ct-DNA on follow-up. The ct-DNA values in these patients with localized recurrent disease was 18 and 56, at 6 months and 9 months respectively. Two patients had widespread metastasis, with progressive disease on imaging, increasing ct-DNA values and were treated palliatively. The ct-DNA values in patients with widespread metastasis was 78445 and 159 with confirmation of recurrence at 9 months.

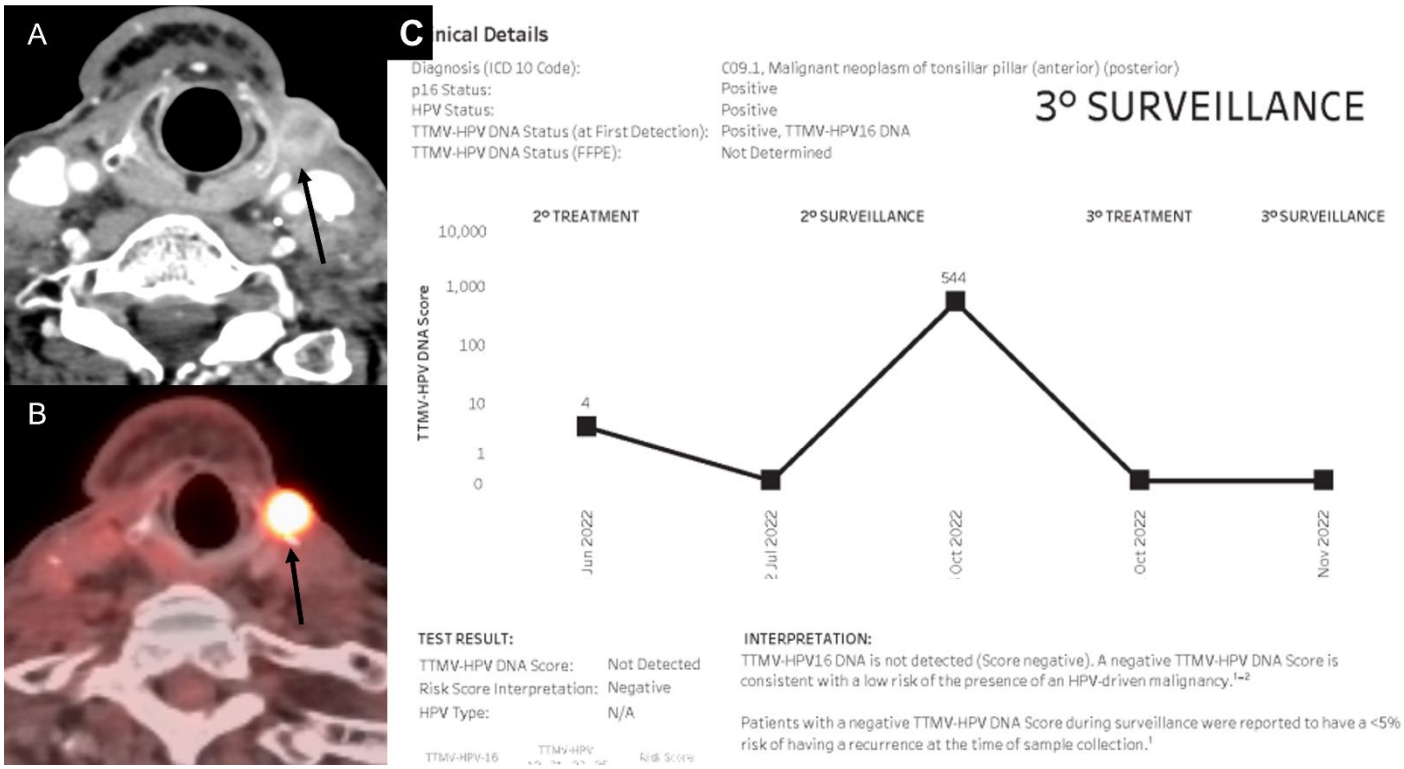

FIG 3. Recurrence of p16+ oropharyngeal SCCa along the left lateral neck with concordant imaging and ct-DNA results. Surveillance contrast-enhanced neck CT (A) and PET-CT (B) in a patient with treated p16+ oropharyngeal SCCa showed nodular enhancement with increase FDG uptake in the left lateral neck (black arrows) concerning for tumor recurrence. Marked increase in values from 0 to 544 was seen on the concurrent ct-DNA test. This was treated with surgical resection (curative intent) with return of ct-DNA values to 0 (negative) on follow-up surveillance.

Overall, the negative predictive value and positive predictive value for ct-DNA was 100% **(Table 1**), with the latter, however, limited by a small sample size (4 positive patients). There were no false-negative ct-DNA cases in this study. There was an increase in the ct-DNA value of two patients on the first post-treatment study, presumed to be secondary to high tumor fragmentation, with negative ct-DNA values on subsequent tests.

Table 1: Results with absolute number and percentages of positive and negative imaging and CT and/or FDG PET-CT (total cohort=34 patients).

	Total cohort (n=34)100%)	ct-DNA	CT/FDG PET-CT	Long-term surveillance or pathology outcome
Concordant negative	22 (*64.7%)*	22 (negative)	22 (negative)	Negative on long-term surveillance
Discordant	8 (*23.5%)*	7 of 8: Negative ct-DNA results and positive findings on imaging		Negative on long-term surveillance
		1 of 8: Positive ct-DNA results and negative imaging		Positive on pathology
Concordant positive	4 (*11.7%)*	4 (positive)	4 (positive)	Positive on pathology
	NPV= 100% PPV=100%			

DISCUSSION

Over the past decade, oropharyngeal SCCa has become the most prevalent cancer associated with HPV in the United States.[1] Despite having much better prognosis compared to non-HPV oropharyngeal SCCa, there is significant risk of recurrence (15-25%) within first five years of definite therapy.[1,2] Circulating tumor DNA (ct-DNA) has emerged as a robust diagnostic tool to detect and quantify HPV positive tumors.[3,5] Conventional ct-DNA could not distinguish HPV DNA, secondary to a wide range of acute or chronic viral infections, from tumor-DNA. However, with recent developments, these tests can precisely and specifically measure cell free tumor tissue modified viral (TTMV)-HPV load using ultrasensitive multianalyte digital droplet PCR assay tests. TTMV-HPV DNA is a unique biomarker released in the bloodstream by fragmentation of malignant epithelial cells with HPV modified DNA.[5-8] Studies suggest that the quantity of HPV ct-DNA shed into plasma varies significantly and is dependent on tumor (including nodal) burden, HPV genomic integration, and other tumor features such as proliferation and vascularization.[5,9] Around 80-90% of patients with oropharyngeal SCCa have detectable levels of ct-DNA at time of diagnosis which is established as a baseline.[5] Low baseline level of ct-DNA is indicative of low tumor HPV copy number and higher rates of HPV genomic integration, both of which are associated with adverse tumor genomic features. In contrast, patients with abundant (>200 copies/ml) ct-DNA levels have favorable prognosis.[5,6] Pre-treatment ct-DNA levels correlate strongly with the overall tumor burden, and rapid clearance of the biomarker after chemoradiotherapy predicts the likelihood of disease control.[9] Some patients

may show transient increase ("spike") after initiation of chemoradiation, likely reflecting early tumour cell destruction and this could have clinical value as a surrogate for early treatment response. The transient elevation in HPV ct-DNA resolves in all patients without recurrent disease.[5,10] Serial increase in ct-DNA values provides stronger correlation with tumor recurrence compared to a single time-point increase in values. Literature on the role of this marker as a tool for surveillance post-definite therapy is still limited. The National Comprehensive Cancer Network (NCCN) guidelines (Version 3.2024) for surveillance includes periodic clinical and imaging exams in the first 6 months post-treatment (short-term) including CT and/or MRI within 3–4 months after surgical treatment for patients with locoregionally advanced disease or with altered anatomy resulting in difficult clinical assessment, in order to establish a new baseline for future comparisons. FDG-PET/CT is the most sensitive imaging modality and is recommended within 3–6 months of definitive radiation or systemic therapy for assessment of treatment response and to identify any residual tumor. There are no consensus guidelines on the frequency and modality of routine long-term (>6 months to 5 years) post-treatment imaging in the asymptomatic patient with wide variability across institutions.[4] Clinical exams, including endoscopic evaluation, has limited sensitivity for early detection of tumor recurrence.[6] Imaging with contrast-enhanced CT and/or PET imaging forms the mainstay with high sensitivity. PET-CT offers a NPV of almost 100%, however, there is a high false positive rate (20-30%) limiting the PPV.[11,12] These imaging exams are limited in specificity secondary to a wide range of factors including scarring, inflammatory changes, and complex appearance of flaps. Although the findings of PET and contrast-enhanced neck CT are concordant in the vast majority of cases, both have limitations when it comes to ambiguous cases.[11] Longitudinal assessment of HPV ct-DNA offers a very sensitive non-invasive study, which, when combined with imaging, can offer almost 100% sensitivity and specificity. Sequential positive results during the surveillance phase have exponentially higher sensitivity for recurrence compared to a single positive result.[6,7] Although studies are limited at present, the sensitivity, PPV, and NPV of these biomarkers exceed that of any imaging exam. The serological tests have the potential for early detection of tumor recurrence and can serve as an alternative for invasive tissue biopsies in cases with ambiguous imaging findings.[6,7] Moreover, the blood tests can potentially decrease the frequency of radiologic and endoscopic exams, for long-term surveillance of HPV positive SCCa. Alternatively, upward trends in the serological marker could prompt an earlier imaging study to localize the site of recurrence. Patients with oligometastatic (1-5 lesions) can be treated with curative intent as compared to palliative therapy for widespread metastasis.[13] Initial studies, though limited in number, have consistently shown 94-100% NPV with low-likelihood of tumor recurrence in the absence

of ct-DNA elevation. [5] The PPV was also very high (around 90-95%) in most studies, with some patients developing a transient spike in ct-DNA without recurrence on longitudinal follow up.[3,8] In our study, there were two cases of HPV equivocal SCCa on p16 immunostaining that had undetectable levels of pre-treatment ct-DNA, which were excluded. P16 immunohistochemistry is the most widely used test for assessment of HPV causation in oropharyngeal SCCa and serves as an easy and economical surrogate for HPV DNA or RNA testing. It is however important to note that tumor may be inaccurately categorized as HPV+ by p16 staining or other HPV assays in up to 20% of cases.[14] Discordance between p16 and HPV DNA or RNA status affects patient prognosis in terms of disease-free and overall survival. Recent studies have shown that patients with discordant oropharyngeal SCCa (p16–/HPV DNA or RNA+ or p16+/HPV DNA or RNA –) had a significantly worse prognosis than patients with p16+/HPV DNA or RNA+ oropharyngeal cancer, however significantly better prognosis than patients with p16–/HPV DNA or RNA– oropharyngeal SCCa.[14] In cases of discordant pre-treatment results, repeat testing with a combination of p16 immunohistochemistry and HPV DNA PCR offers high sensitivity for definitive assessment of tumor HPV status. This is recommended where HPV status might influence patient care, especially in geographical regions with low HPV-attributable fractions.[5] Detection of HPV oncogene (HPV E6/E7 mRNA) transcripts using an amplification-based method constitutes the current gold standard to identify the etiological role of HPV in oropharyngeal SCCa. However, considering the comparatively laborious methodology and high cost of these tests, simple HPV DNA PCR with p16 immunohistochemistry serve as a reasonable alternative.[15] In the study by *Chera et al* with 115 subjects, 15 patients developed recurrence. In this subset, there were four patients where neck imaging failed to detect tumor recurrence, as the recurrence was outside the region being imaged (neck or chest) in all four cases .[8] The findings in our study is concordant with other published literature. Although limited by a small sample size, this study had a negative predictive value of 100%. It has been less than one year since the tests have been incorporated into routine clinical practice at our center. Over the next few years, we expect larger series comparing imaging and serological tests for surveillance of oropharyngeal SCCa. Discussion of ct-DNA is now an integral part of multidisciplinary tumor boards and offers a great opportunity to for detection of early and small volume tumor recurrence. The economic value of "liquid biopsy" testing is affected by not only the sensitivity and specificity of ct-DNA, but also the clinical utility of the study. Clinical utility can be demonstrated by change in patient outcomes or clinical practice guidelines, such as decreased frequency of physical and imaging exams and associated financial burden of outpatient surveillance care.[5-8]

CONCLUSION

Longitudinal monitoring of HPV ct-DNA for post-treatment surveillance of p16+ oropharyngeal SCCa can accurately detect disease recurrence with a potential to decrease the frequency of physical and imaging exams and modification of the surveillance guidelines . The neuroradiologist should integrate the results of serological tests into their interpretation of surveillance imaging. Additional studies with larger cohorts and longer time course of follow up are however needed for further validation of these biomarkers.

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SUPPLEMENTAL FILES

Supplemental Table 1: Excel sheet with details on patient age, gender, tumor stage, HPV-subtype, treatment, ct-DNA longitudinal values, pathology, concordant and discordant status

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Age	Gend	Location	TNM	HPV su	Treatment	Pre-treatr	1st follow	2nd follov	3rd-5th fo	Imaging (fo	Path for re	Concordant/Dis
2	69	M	Right BOT	T4N3M0	16	Chemoradiation	2327	2531	29	0	Negative	N/A	Concordant
3	63	M	Right tonsil	T1N1M0	16	Chemoradiation	63	0	0	0	Negative	N/A	Concordant
4	59	M	Right tonsil	T2N1M0	16	TORS + ND	20	0	0	0	Negative	N/A	Concordant
5	54	F	BOT	T1N2M0	16	Chemoradiation	189	9	0	0	Negative	N/A	Concordant
6	58	F	Right BOT	T1N0M0	16	Chemoradiation	13	0	40	98-159	Positive	Positive	Concordant
7	82	M	BOT	T4N2M0	16	Chemoradiation	212	0	0	0	Negative	N/A	Concordant
8	47	M	Left tonsils	T2N1M0	16	TORS +ND	7	0	0	0	Negative	N/A	Concordant
9	71	M	BOT	T4N2M0	16	Chemoradiation	632	0	0	0	Negative	N/A	Concordant
10	67	M	Rt BOT	T1N1M0	16	TORS + BND	260	0	0	0	Negative	N/A	Concordant
11	78	F	BOT	T4N2M0	16	Chemoradiation	104	0	0	0	Negative	N/A	Concordant
12	59	F	Lateral wall	T3N2M0	16	Chemoradiation	8874	606	0	0	Positive	N/A	Discordant
13	68	M	Left BOT	T4N2M0	16	Chemoradiation	544	0	0	56	Positive	Positive	Concordant
14	62	F	Left BOT	T1 N1 M0	16	TORS+ND	11	0	0	0	Negative	N/A	Concordant
15	75	M	Midline BOT	T1 N2 M0	16	TORS +CRT	5020	0	0	0	Negative	N/A	Concordant
16	60	M	Right tonsils	T2N1M0	16	Wild-filed tonsilee	170	0	0	0	Positive	N/A	Discordant
17	64	F	Left tonsils	T4N2M0	16	Chemoradiation	66	0	0	0	Positive	N/A	Discordant
18	48	F	BOT	T1N0M0	16	TORS +ND	22	0	4	0	Negative	N/A	Concordant
19	76	M	Right Tonsils	T1 N2M0	16	Chemoradiation	30511	0	18	27-75	Positive	Positive	Concordant
20	69	M	BOT	T4N3M0	16	Chemoradiation	2327	2531	20	0	Negative	N/A	Concordant
21	49	M	Left tonsils	T1N1M0	16	Chemoradiation	222	9	0	0	Negative	N/A	Concordant
22	70	M	Left tonsils	T1N1M0	16	Wild-filed tonsilee	143	0	0	0	Negative	N/A	Concordant
23	63	M	Left BOT	T4N2M0	16	Chemoradiation	135765	17	0	0	Negative	N/A	Concordant
24	72	M	Left tonsils	T4N2M0	16	Chemoradiation	101	0	0	0	Negative	N/A	Concordant
25	67	M	Left tonsils	T2N1MX	16	Chemoradiation	167	0	0	0	Negative	N/A	Concordant
26	49	M	Right tonsils	T4N2bM0	16	Chemoradiation	12	0	0	0	Negative	N/A	Concordant
27	71	M	Left tonsil	T2N2M0	16	Chemoradiation	143	0	0	0	Negative	N/A	Concordant
28	55	M	Rt BOT	T1N2M0	16	TORS +ND	13	0	0	0	Positive	N/A	Discordant
29	73	F	Left tonsils	T2N2M0	16	TORS+ND	479	0	0	0	Negative	N/A	Discordant
30	53	F	Left tonsils	T3N0Mo	16	Chemoradiation	9404	415	0	0	Negative	N/A	Concordant
31	58	M	BOT	T3N1M0	16	TORS+BND	307	0	0	0	Negative	N/A	Concordant
32	50	M	Left tonsils	T1N1M0	16	TORS +ND	222	9	0	0	Positive	N/A	Discordant
33	82	M	Left BOT	T4N2M0	16	Chemoradiation	212	0	0	0	Positive	N/A	Discordant
34	62	M	BOT	T3N1M0	16	Chemoradiation	2428	0	10900	78445	Positive	Positive	Concordant
35	58	F	Right BOT	T1N0M0	16	TORS +ND	13	40	98	152	Negative	Positive	Discordant
36													
37	ND=	Neck Dissection		BND= Bilateral neck dissection			CRT=Chemoradiation						