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A Highly Sensitive Real-Time RT-PCR Assay for Detection of HPV-16 E7 Transcript in Head and Neck Squamous Cell Carcinomas

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Abstract

Highly sensitive HPV-16 assays suitable for clinical settings are critical to screen patients for emerging HPV targeted therapies. In this light, we developed a novel TaqMan RNA PCR assay for qualitative detection of HPV-16 E7 gene transcript using formalin-fixed and paraffin-embedded (FFPE) samples. Furthermore, the assay was compared to another SYBR green RNA PCR assay, a DNA genotyping assay and a composite method consisting of p16 IHC and HPV DNA genotyping assay. The results of 82 clinical HNSCC samples demonstrated that the TaqMan RNA assay detected 19% of the negative samples by the SYBR green RNA assay, 27% by the DNA genotyping assay and 24% by the composite assay while capturing 100% of the positive samples detected by the SYBR green RNA assay or the composite method. Meanwhile, 100% of the negative samples by the TaqMan assay were also negative by at least two other assays. To interrogate the discordant samples that were positive by the TaqMan RNA assay but negative by the SYBR green RNA assay, the analytical sensitivity of the two assays was evaluated using a simulated HPV-16 positive RNA sample panel. The result illustrated that the TaqMan RNA assay has sensitivity 10-fold or greater than that of the SYBR green RNA assay. Our study indicates that 1) laboratory-developed HPV-16 RNA PCR assays may have significant variability in performance even with similar methodologies. 2) A notable portion of HPV-16 negative samples by the methods reported from HNSCC clinical studies could be positive for the viral transcripts. These findings highlight the urgency of developing more accurate and reliable HPV-16 assays to select patients that are most likely to benefit from treatment regimens of HPV-targeted therapies.

Keywords: HPV-16 Assays**,** RNA, Therapy

Introduction

High-risk human papillomavirus (HPV), particularly subtype 16 (HPV-16), is established as one of the drivers for head and neck squamous cell carcinomas (HNSCC) [1]. Interestingly, numerous studies have identified that HPV positivity in HNSCC is correlated with favorable prognosis [2,3], which indicates that HPV-related HNSCC is a biological and clinical variant of the disease. Clinical trials are evaluating possible de-escalation of therapies in this disease population due to an improved treatment sensitivity and overall survival rate of the patients [4]. The value in prognosis and its influence in patient management accelerates the inclusion of HPV testing in clinical practice. The College of American Pathologists has recently recommended routine HPV testing for oropharyngeal squamous cell carcinoma, a subset of HNSCC [5].

Recently the need for HPV testing has become even more compelling with the emergence of tumor antigen-targeted immunotherapies for advanced HNSCC that is incurable by chemotherapy [6]. HPV-16 accounts for a majority of HPV positive tumors and expression of its E6 and E7 oncoproteins is absent in healthy tissues [7], which makes it an

attractive target for anti-tumor medicines [8]. Accurate detection of HPV-16 is critical to select patients who are most likely to benefit from these target-specific treatment approaches.

Despite the significance of reliable determination of HPV status, there is yet to be a standard method for HPV or HPV-16 diagnosis in HNSCC. Methods of HPV testing across laboratories vary considerably not only in assay technologies, but also in the detection targets. Since active expression of E6 and E7 genes of HPV is required for carcinogenesis, detection of E6 or E7 transcripts by RNA PCR in fresh frozen tumor tissue is considered as the gold standard method [9]. However, requirement of fresh frozen tissue for some previously reported RNA assays [10] severely limits its application in clinical settings. Formalin-fixed paraffin-embedded (FFPE) tissue is a more commonly available sample type, but higher RNA destruction and fragmentation and subsequent decreased sensibility is a constant concern. A RNA in-situ hybridization (ISH) assay using RNA scope probes has been reported for high-risk HPV E6/E7 detection in FFPE tissue and achieved high sensitivity and specificity [11, 12], but its high complexity might be a hurdle for widely adoption in clinical studies. On the other hand, detection of a surrogate marker, p16 protein, by immunohistochemistry (IHC) in FFPE tissue is adopted in many clinical laboratories. However, it was reported that a significant proportion of patients that exhibited p16 positivity by IHC was not concordant for HPV DNA ISH or DNA PCR assays [13,14]. While HPV DNA PCR may be more specific than p16 IHC and able to differentiate between HPV-16 and other HPV subtypes, notable false positivity of such assays has been reported [15]. In light of these issues, a composite method consisting of both p16 IHC and HPV DNA assay was proposed, and a relatively high concordance to the RNA assay was reported [15].

The purpose of this study was to develop a sensitive TaqMan HPV-16 RNA PCR assay for FFPE tumor tissue and compare its performance to a SYBR green-based RNA PCR assay adapted from a recently published method that also detects HPV-16 E7 transcript [16]. In addition, a composite assay which combined the results of both p16 IHC and HPV DNA was performed using the same sample set for direct comparison with the RNA assays.

Materials and Methods

Patients and tumor samples

A total of 95 archival FFPE tissue blocks of HNSCC patients were acquired (Avaden Biosciences, Seattle, WA). De-identified demographic and pathological information (age, gender, sites of biopsy or resection, diagnosis, and staging) of all the patients and the patients whose samples were tested in the study are summarized respectively as “Overall” and “Evaluated” sections in Table 1.

Table 1. Characteristics of the clinical sample cohort

	Overall		Evaluated		
	n=95		n=82		
	value	%	value	%	
Mean age (years)	61.5		59.5		
Gender					
Male	74	78%	64	78%	p = 0.98
Female	21	22%	18	22%	
Site					
Oropharynx	68	72%	65	79%	p = 0.42
Larynx	24	25%	16	20%	
Other Sites	3	3%	1	1%	
Tumor Grade					
I	35	37%	31	38%	p = 1.00
II	22	23%	19	23%	
III	22	23%	18	22%	
IV	16	17%	14	17%	
cT stage					
T1	40	42%	36	44%	p = 0.98
T2	34	36%	30	37%	
T3	15	16%	11	13%	
T4	6	6%	5	6%	

The samples were analyzed by two RNA PCR assays, a p16 IHC assay, and an HPV DNA genotyping assay as described in the study workflow (Fig 1): 1) For pre-analytical assessment, one section of 4 µm thickness from each block was stained with hematoxylin and eosin (H&E) and reviewed by a pathologist for sample quality control and tumor cellular content analysis. Thirteen samples were excluded due to low amount or poor quality of the tumor tissue. 2) A composite method which combined the results from an p16 IHC and a HPV DNA assay was conducted for the remaining 82 samples and the HPV-16 status of each sample was identified using an algorithm (Fig 2) similar to the approach previously published [15]. 3) all 82 samples were tested with the two HPV-16 RNA assays. For the RNA assays, HPV-16 status of the samples was identified based on the Cq value generated.

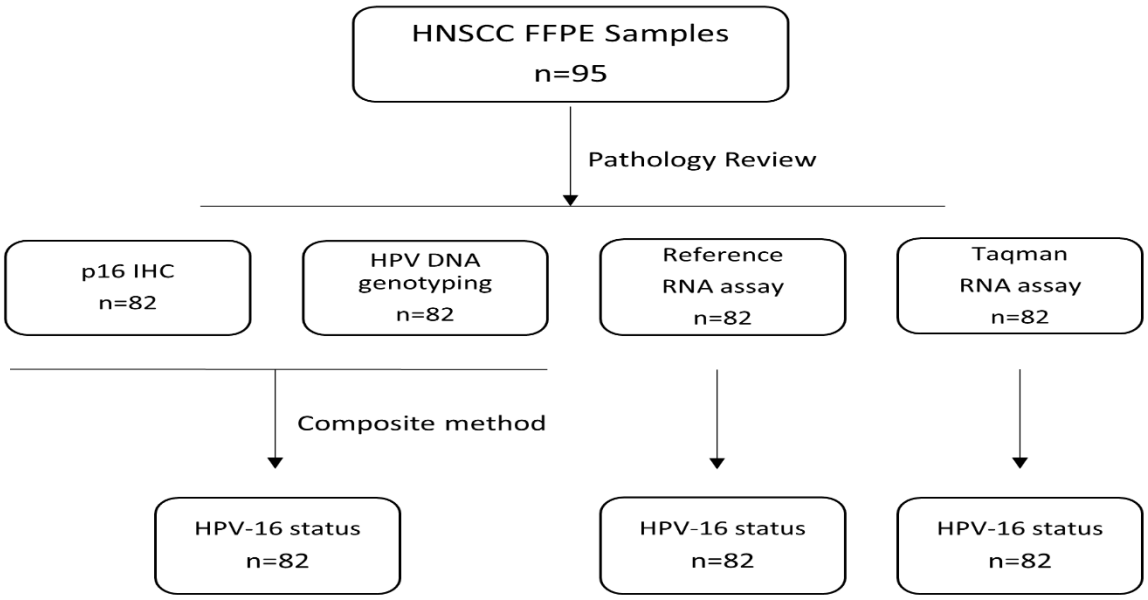

Fig 1. Study workflow.

95 FFPE blocks of HNSCC were reviewed by a pathologist for quality and tumor cellular content. p16 IHC and HPV DNA genotyping assay were conducted as a composite method to determine HPV-16 status of the 82 samples that passed the review. The same sample set was also tested with two RNA PCR assays for the HPV-16 E7 transcript.

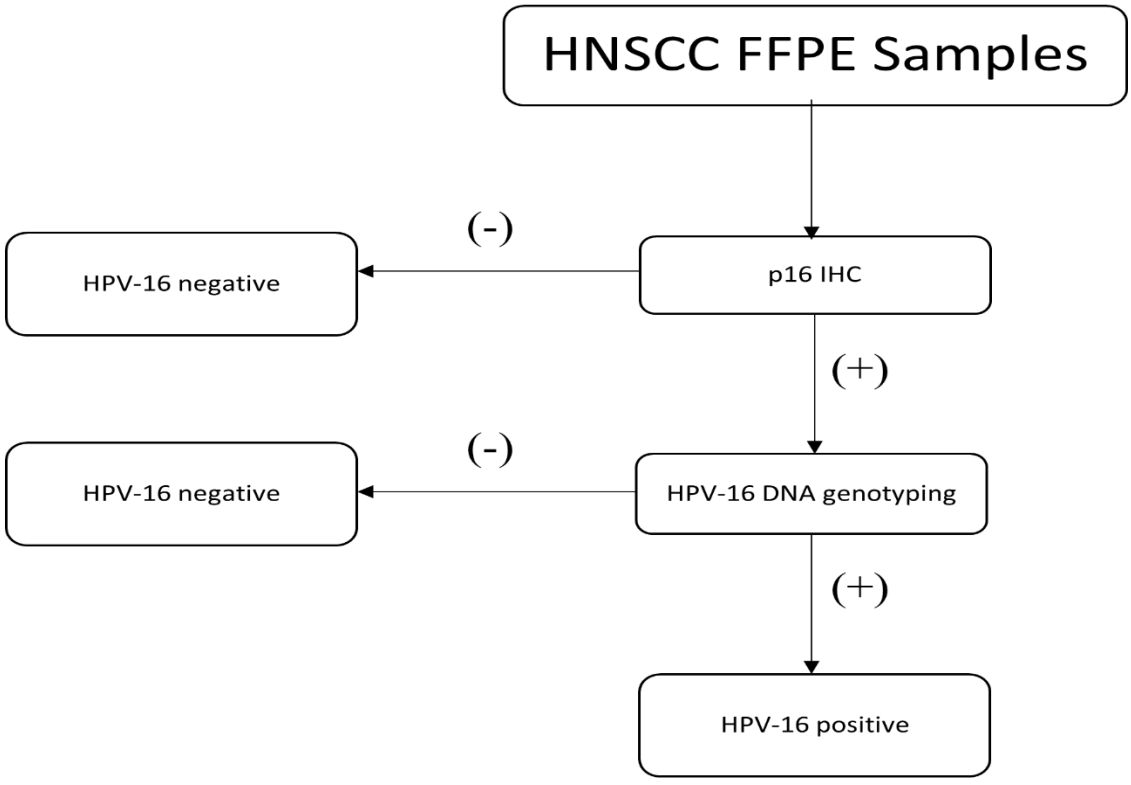

Fig 2. HPV-16 identification algorithm.

The samples with negative p16 IHC staining are considered HPV (including HPV-16) negative. The p16 IHC-positive samples that also yielded HPV-16 specific signal by DNA genotyping are considered as HPV-16 positive, while the p16 IHC-positive samples that were negative for HPV-16 signal by DNA genotyping are remained as negative.

Immunohistochemistry for p16

The p16 IHC was carried out with a commercial kit CINtec Histology (Roche, Tucson, AZ) in a CLIA laboratory (NeoGenomics, FL) for assessment of the p16INK4a protein in FFPE HNSCC tissues. The assay was performed on a VENTANA BenchMark ULTRA instrument (Roche, Tucson, AZ). A HNSCC sample with a high p16 expression was used as a positive control. For the negative control, the primary antibody was omitted from the staining procedure. The positive and negative controls were included for each instrumental run. The p16 IHC stained slides were reviewed and scored by a pathologist (A.K.). A sample was scored positive when greater than 75% of the cells with diffused nuclear and cytoplasmic staining were in the tumor region.

HPV DNA Genotyping Assay

The HPV-16 DNA assessment was conducted using an HPV DNA genotyping test which was developed at a CLIA laboratory (NeoGenomics, FL). In brief, HPV subtype-specific primers were designed to anneal to DNA in early protein genes (E5–E7) and PCR products of various sizes for different HPV subtypes were generated. Specific HPV subtypes were identified by determining the size of PCR products using ABI3730xl Genetic Analyzer (Thermo Fisher, Foster City, CA). Six high-risk types, 16, 18, 31, 33, 45, 58, and two low-risk types in combination 6/11, could be identified by the fragment analysis, which covers approximately 95% of cancer-related HPV.

SYBR green RNA PCR assay

A SYBR green RNA PCR assay adapted from a recent report [16] was used to assess HPV-16 E7 transcript as a reference method. The oligo primers for the assay were purchased from Thermo Fisher (Foster City, CA). After extraction of RNA from five sections of 5µm thickness using the High Pure FFPE RNA Isolation kit (Roche, Indianapolis, IN), a reverse transcription reaction was done by using the High-Capacity RNA to cDNA Reverse Transcription Kit (Thermo Fisher, Foster City, CA) with 30 ng RNA in a 20 µL volume of reaction. Real-time PCR was carried out with 2 µL cDNA by using Power SYBR Green PCR Master Mix (Thermo Fisher, Foster City, CA) and 500 nM each primer in a 10 µL volume of reaction. The sequences of the primers used are as follows: HPV16 E7 forward primer: 5' GAACCGGACAGAGCCCATTA 3'; HPV16 E7 reverse primer: 5’ACACTTGCAACAAAAGGTTACA 3'; The PCR assay conditions included: 95°C for 10 minutes, followed by 40 cycles of amplification (95°C for 10 seconds, 58°C for 15 seconds and 60°C for 15 seconds). A similar SYBR Green PCR assay for β-actin gene was set up in a separate well of the same 384-well plate as control for sample input RNA quality and PCR process. Samples that did not yield Cq value for the control assay were considered invalid and were excluded from data analysis

Development of a TaqMan RNA assay for HPV-16 E7 transcript

A TaqMan RNA PCR assay was designed and developed for detection of HPV16 E7 transcript in FFPE samples. Primers and probes were designed by using the NCBI (National Center for Biotechnology Information) reference sequence NC_001526.4:7604-7900 of Human papillomavirus type 16. The amplification products were designed to be <120 bp in length to ensure optimal performance for highly fragmented RNA from FFPE samples. Primers and probes were purchased from Thermo Fisher (Foster City, CA) in a premixed 20x or 40x solution. The sequences of the primers and probe used are as follows: HPV16 E7 forward primer: 5' GAGGATGAAATAGATGGTCCAGCT 3'; HPV16 E7 reverse primer: 5' ACAACCGAAGCGTAGAGTCACA 3'; FAM-MGB probe: 5' ACAAGCAGAACCGGACAG 3'. After extraction of RNA from five sections of 5µm thickness using the High Pure FFPE RNA Isolation kit (Roche, Indianapolis, IN), reverse transcription reactions were conducted by using a High-Capacity RNA to cDNA Reverse Transcription Kit (Thermo Fisher, Foster City, CA) with 30 ng RNA in a 20 µL volume of reaction. After a proprietary pre-amplification protocol (Almac Diagnostics) with primers for HPV-16, TOP1 and β-actin genes, real-time PCR for HPV-16 was carried out in a 10µL reaction volume that included 1x TaqMan® Fast Advanced Master Mix (Thermo Fisher, Foster City, CA), 1x primer/probe and 2.5µL pre-amplification product, followed by a thermal profiling protocol: 50°C for 2 minutes, 95°C for 20 seconds, then 40 cycles of amplification (95°C for 1 seconds and 60°C for 20 seconds). A similar real-time PCR reaction was set up for TOP1 and β-actin gene respectively in separate reaction wells of the same 384-well plate as controls for sample RNA quality and as an in-process PCR control. Samples that did not yield appropriate Cq values for either of the reference genes were considered invalid and were excluded from data analysis.

Evaluation of analytical sensitivity and specificity of the RNA assays

To evaluate and compare the analytical sensitivity of the two RNA assays, HPV-16 positive RNA from formalin-fixed pellets of SiHa cell line (AddexBio, San Diego, CA) was serially diluted with HPV-16 negative RNA (universal human RNA, Agilent, CA) to make a panel of 15 diluted samples with a same total RNA concentration and decreasing fraction of HP-16 positive RNA (neat, 1:1, 1:4, 1:8, 1:16, 1:32, etc., Table 2).

Table 2. Analytical sensitivity of the two RNA assays

Sample	HPV-16 RNA fraction in total RNA	Samples Positive (%) TaqMan Assay	Samples Positive (%) Reference SYBR Green Method
Neat	1	12 (100%)	12 (100%)
Dilution 1	1/2	12 (100%)	13 (100%)
Dilution 2	1/4	12 (100%)	14 (100%)
Dilution 3	1/8	12 (100%)	15 (100%)
Dilution 4	1/16	12 (100%)	16 (100%)
Dilution 5	1/32	12 (100%)	17 (100%)
Dilution 6	1/64	12 (100%)	18 (100%)
Dilution 7	1/128	12 (100%)	19 (100%)
Dilution 8	1/256	12 (100%)	20 (100%)
Dilution 9	1/512	12 (100%)	21 (100%)
Dilution 10	1/1024	12 (100%)	22 (100%)
Dilution 11	1/2048	12 (100%)	23 (100%)
Dilution 12	1/4096	12 (100%)	10 (83%)
Dilution 13	1/8192	12 (100%)	6 (50%)
Dilution 14	1/16384	12 (100%)	4 (33%)
Dilution 15	1/32768	12 (100%)	3 (25%)

Both RNA assays were tested using the panel with 12 replicates for each dilution level and 30 ng RNA input per reaction. The lower limit of detection (LLOD) was defined as the lowest level of HPV-16 positive RNA (in terms of its fraction in the total RNA) that 100% of the 12 replicates yielded an acceptable Cq value to make a positive call.

Analytical specificity of each assay was tested with 10 replicates of the HPV16 negative RNA (human universal RNA, Agilent, CA) using 30 ng input**.**

Results

Patient Characteristics and HPV-16 diagnosis by the composite method of p16 IHC plus HPV DNA genotyping assay

The demographic and pathological characteristics of the 82 patients with samples that passed the pathological review were summarized in Table 1. The patient characteristics are consistent to the overall 95-sample set.

All 82 samples were tested by the p16 IHC assay. 39 samples (47.6%) were positive for p16 staining, with stained cells ranging from 75% to 100% and a median of 95%. 43 samples (52.4%) were negative (0 to 5% stained cells, median 0%). Four samples exhibited a borderline stained result (75%). Representative p16 IHC stains are shown in Fig 3. The sample set was also tested by the HPV DNA genotyping assay. 37 samples (45.1%) were positive and 45 samples (54.9%) were negative for HPV-16 respectively.

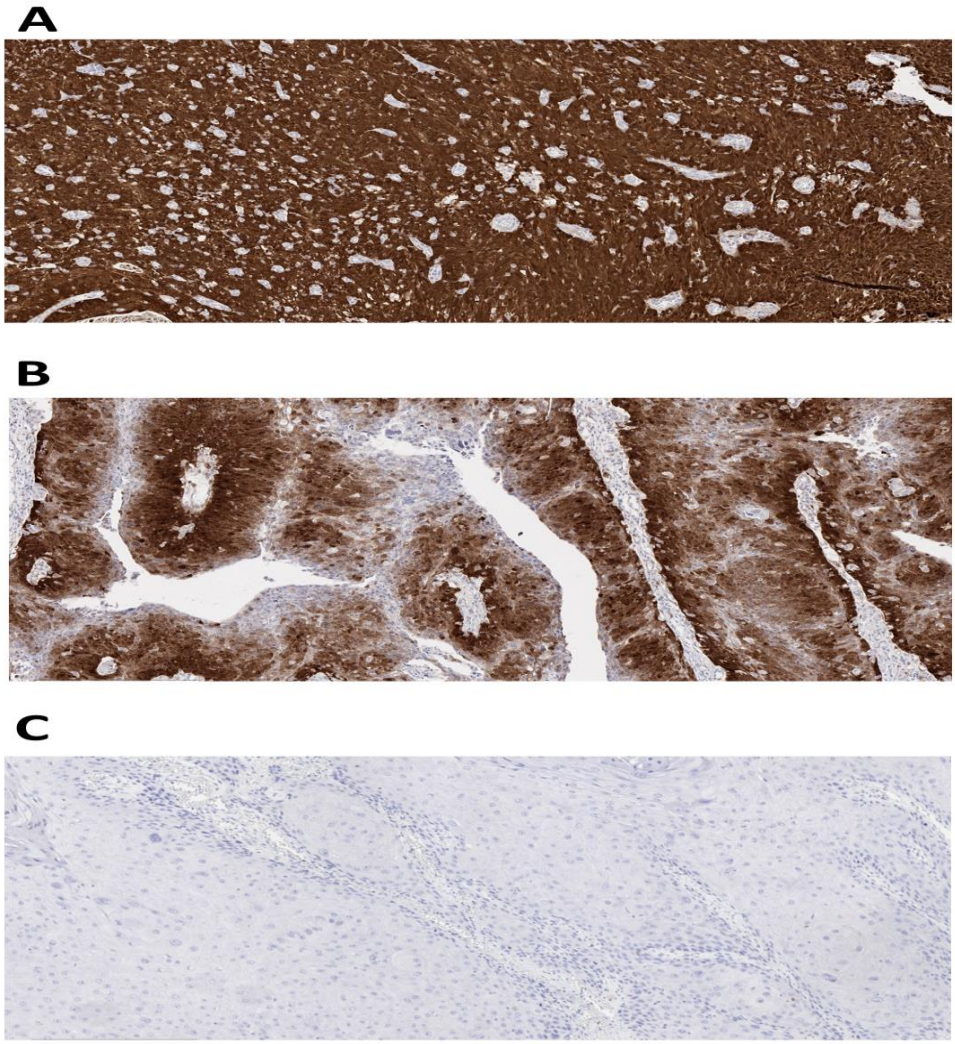

Fig 3. p16 IHC staining on HNSCC tumors. The percentage of cells with diffused nuclear and cytoplasmic staining of p16 in tumor regions are used to determine HPV status of the sample. Samples show distinct staining patterns close to 100% are positive (A) and close 0% are negative (C). Samples exhibited staining patterns of 75-80% cells are borderline (B).

The results of the p16 IHC and HPV DNA genotyping assay were further analyzed with the algorithm in Fig 2. Through use of this composite method, 7 samples previously positive via p16 IHC alone and 5 samples previously positive via HPV DNA genotyping alone were considered negative, which yielded 32 (39%) positive HPV-15 samples and 50 negative samples (61.0%).

Reference RNA PCR assay is comparable to the composite method

With the reference SYBR green RNA assay, HPV-16 E7 transcript was detected in 35 (43%) samples and 47 (57%) samples were negative. The overall percent concordance between the reference assay and the composite method is 91% (Table 3A).

Table 3. Comparison of various HPV-16 assays

A.

	Composite +	Composite -	
Reference assay +	30	5	35
Reference assay -	2	45	47
	32	50	

B.

	Reference assay +	Reference assay -	
TaqMan assay +	35	9	44
TaqMan assay -	0	38	38
	35	47	

C.

	DNA PCR +	DNA PCR -	
TaqMan assay +	32	12	44
TaqMan assay -	5	33	38
	37	45	

D.

	Composite +	Composite -	
TaqMan assay +	32	12	44
TaqMan assay -	0	38	38
	32	50	

TaqMan RNA assay is more sensitive than the other assays

Using the newly designed TaqMan RNA PCR assay, HPV16 E7 transcript was detected in 44 of 82 samples (54%). Nine samples positive by the TaqMan assay were negative by the reference RNA assay (Table 3B), resulting in a NPV of 81% (38/47) vs the reference RNA assay. Thirty-eight samples (46%) were negative by the TaqMan assay and all of them were negative by the SYBR green RNA assay too, and a PPV of 100% (35/35) vs the reference RNA assay. The overall concordance between the two RNA assays is 89%.

The TaqMan assay was also compared to the HPV DNA genotyping assay directly (Table 3C). 12 of 44 positive samples by the TaqMan assay yielded negative result for the DNA PCR assay, while 5 of 38 negative samples by the TaqMan assay were positive by the DNA genotyping. The overall concordance between the two assays is 79%.

Compared to the composite method (Table 3D), 12 samples that were positive by the TaqMan assay were negative by

the composite method, while all the negative samples by the TaqMan assay were negative by the composite method. The concordance between the two methods is 85%.

P16 IHC result was not compared to that of the TaqMan RNA assay since the IHC assay alone is not specific for HPV-16 subtype.

Direct comparison of HPV-16 results for each sample (Table 4) from the four assays confirmed that all the negative samples by TaqMan assay (upper part of the table) were negative by at least two other assays, while various number of negative samples by each of the other assays were positive by the TaqMan assay (lower part of the table).

Table 4. HPV-16 results from the four assays

Sample ID	TaqMan	Reference	Composite	DNA genotyping
S002	-	-	-	-
S003	-	-	-	-
S006	-	-	-	-
S007	-	-	-	-
S008	-	-	-	-
S009	-	-	-	-
S012	-	-	-	-
S013	-	-	-	-
S014	-	-	-	-
S015	-	-	-	-
S016	-	-	-	-
S017	-	-	-	-
S021	-	-	-	-
S022	-	-	-	-
S027	-	-	-	-
S028	-	-	-	-

S032	-	-	-	-
S034	-	-	-	-
S040	-	-	-	+
S042	-	-	-	-
S043	-	-	-	+
S044	-	-	-	-
S045	-	-	-	+
S048	-	-	-	-
S054	-	-	-	+
S058	-	-	-	-
S059	-	-	-	-
S060	-	-	-	-
S064	-	-	-	-
S065	-	-	-	-
S067	-	-	-	-
S068	-	-	-	-
S069	-	-	-	-
S072	-	-	-	-
S073	-	-	-	-
S075	-	-	-	-
S081	-	-	-	+
S082	-	-	-	-
S001	+	+	+	+

S004	+	+	-	-
S005	+	+	+	+
S010	+	+	+	+
S011	+	+	+	+
S018	+	+	+	+
S019	+	+	+	+
S020	+	-	+	+
S023	+	+	+	+
S024	+	-	+	+
S025	+	+	+	+
S026	+	+	-	-
S029	+	+	+	+
S030	+	+	+	+
S031	+	+	+	+
S033	+	+	+	+
S035	+	-	-	-
S036	+	+	+	+
S037	+	+	+	+
S038	+	+	+	+
S039	+	+	+	+
S041	+	+	+	+
S046	+	+	+	+
S047	+	-	-	-

S049	+	+	+	+
S050	+	-	-	-
S051	+	+	+	+
S052	+	+	-	-
S053	+	+	-	-
S055	+	-	-	-
S056	+	+	+	+
S057	+	+	+	+
S061	+	+	+	+
S062	+	+	+	+
S063	+	+	+	+
S066	+	-	-	-
S070	+	+	+	+
S071	+	+	+	+
S074	+	-	-	-
S076	+	+	+	+
S077	+	-	-	-
S078	+	+	-	-
S079	+	+	+	+
S080	+	+	+	+

The quantitative Cq values of the samples from the TaqMan RNA assay were compared with that of the reference RNA assay. A total of 35 samples yielded Cq values for both assays. While the Cq values by the TaqMan assay were highly correlated to that by the reference assay (Pearson's r =0.94, Fig. 4) across the samples, the Cq values of the TaqMan RNA assay were significantly lower than that of the reference RNA assay in the pair-wise comparison ($p < 0.0001$, Fig. 5).

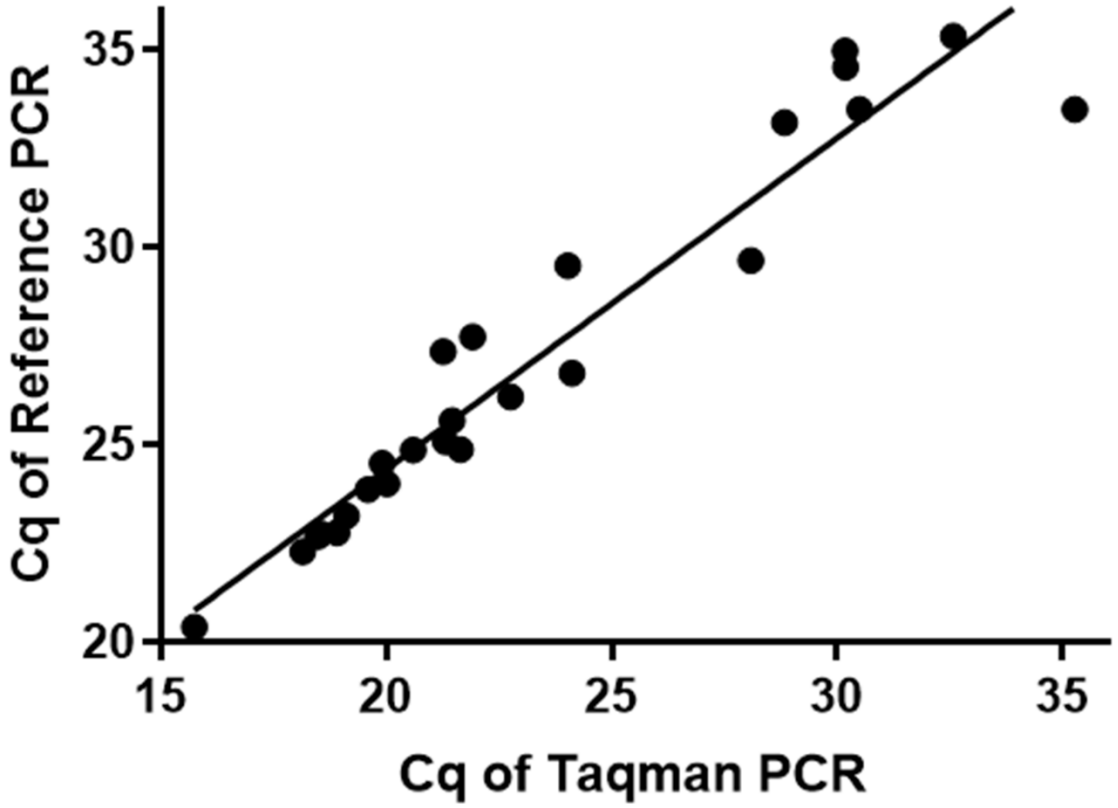

Fig 4. Correlation of Cq values from RNA PCR assays. Cq values from the TaqMan RNA assay and the reference assay for each of 35 positive samples are plotted for correlation. Pearson correlation coefficient is 0.96, $p<0.0001$

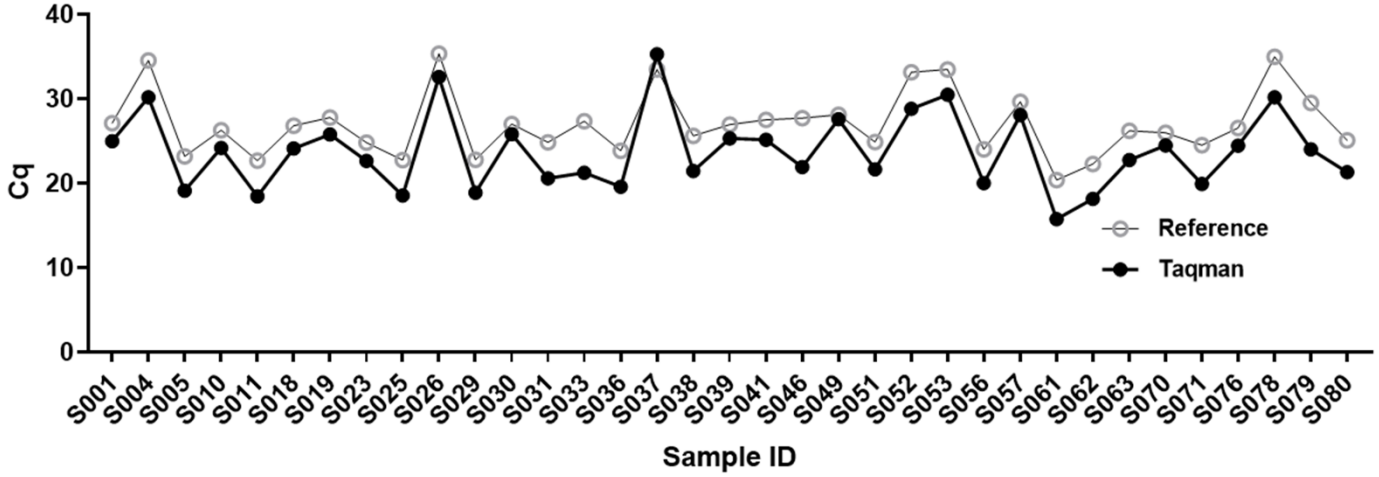

Fig 5. Pair-wise comparison of Cq values of RNA PCR assays. Cq values from the TaqMan RNA assay are compared to the reference RNA assay for each of 35 positive samples by pair-wise t test. Cq values of the TaqMan assay is significantly lower than that of the reference assay, $p<0.0001$

TaqMan RNA assay shows higher analytical sensitivity than the reference RNA assay

To interrogate the discordance seen for clinical samples positive by TaqMan RNA assay and negative by the reference RNA assay, the analytical sensitivity of the two assays was evaluated using a simulated HPV-16 positive RNA sample set. While the reference RNA assay detected 100% of the sample replicates down to 1:2,048 fraction of HPV-16 positive RNA in the total RNA, the TaqMan assay detected 100% of the sample replicates with 1:32,768 fraction of HPV-16 positive RNA (Table 2). The results indicates that the TaqMan assay has a sensitivity approximately 10-fold greater or more than the reference RNA assay.

Both the TaqMan assay and the reference RNA assay yielded no amplification signal in the 10 negative RNA samples, which demonstrated the high specificity of the two assays.

Discussion

The active transcription of HPV E6 and E7 genes, which leads to synthesis of important oncogenic proteins, is directly related to carcinogenesis in HNSCC. HPV-16 is the most prevalent subtype associated with HNSCC [17]. Therefore, HPV-16 transcripts in tumor tissue have been recognized as a critical biomarker for precision medicine. One major challenge in detecting HPV gene transcripts is the highly fragmented RNA from FFPE samples. The challenge is further exacerbated by the insufficient tumor content that is frequently encountered in clinical samples. To address these challenges, the new TaqMan assay that we developed was specifically designed for amplification of less than 120 bp RNA fragments, and a 10-cycle pre-amplification step was included in the protocol to increase detection sensitivity for FFPE-derived RNA with low amount and/or low quality.

As part of the evaluation process, we compared the newly designed TaqMan assay to a SYBR green-based RNA assay adapted from a recently published RNA detection method that also targets the HPV-16 E7 transcript. The comparison revealed a strong correlation of the Cq values (Pearson's $R = 0.94$, $p < 0.001$) between the two assays. More importantly, it was observed that the Cq values from the newly designed assay were lower than the reference assay by pair-wise comparison ($p<0.001$) when the RNA input was identical, indicating that the new assay is more sensitive in detection of the E7 transcript. Indeed, all the samples (35 of 35, 100%) that were positive by the reference assay were also positive through the new assay, while nearly 20% of the samples (9 of 47, 19.1%) that exhibited no amplification signal by the reference assay were positive through the new TaqMan assay. A sensitivity analysis using a simulated HPV-16 RNA panel confirmed the higher sensitivity of the TaqMan assay observed in the clinical sample testing. One of the limitations of this study is that no alternate reference method exists to confirm these discordant samples are "true positives". Although multiple RNA PCR assays for detection of HPV transcripts were reported before [10, 16, 18, 19], the assays were developed by individual research groups and differed considerably in the targeted gene transcripts, critical reagents and reaction setups. Direct comparison of various HPV-16 RNA PCR assays using simulated RNA sample panels and controlled clinical sample sets have been rarely reported. Our study illustrated that laboratory-developed HPV-16 RNA PCR assays may have significant variability in performance when cross-compared to other similar methodologies. As such, it is critical to conduct a thorough verification of the analytical performance before the selection of a HPV RNA assay for HPV diagnosis of clinical samples.

Results from the two RNA assays were also compared to a composite method of p16 IHC and HPV DNA genotyping assay. For the 32 samples that were positive by the composite method, the TaqMan assay and reference RNA assays were also positive. For the 50 negative samples identified by the composite method, the viral transcripts were detected in 12 samples (24%) by the TaqMan assay and in 5 samples (10%) by the reference RNA assay. If one assumed the results by the reference RNA assay are accurate given there are no gold standard assays, the composite method would have a sensitivity of 86% (30/35) and a specificity of 96% (45/47). The sensitivity further decreased to 73% (32/44) when the TaqMan assay was used as reference. Smeets et al. [15] compared a similar composite method to an in-house designed RNA PCR assay and reported a sensitivity of 100% and a specificity of 100% for the composite. Laboratory-developed RNA assays are often used as a reference in these type of comparison studies. As such, the analytical performance of the RNA assays has a paramount influence on the observed sensitivity and specificity for the assays being evaluated. This may in part account for the lower sensitivity of the composite method shown in our study. The various p16 IHC and HPV DNA genotyping assays used by different research groups might also contribute to the observed difference. With these limitations in mind, one important finding from our work is that around 10%-30% of HPV-16 negative samples by either DNA genotyping assay or a composite method could be positive for the viral transcripts. If validated, this finding may have a profound impact on the decision-making process of treatment regimens and patient enrollment for novel HPV-targeted immunotherapies. False-negative HPV-16 results will exclude patients that might have been eligible for inclusion onto trials or treatment regimens of such therapies, for which they may derive significant benefit. It highlights the urgency of developing more sensitive and reliable methods for accurate diagnosis of HPV in HNSCC and other HPV related cancer types.

In summary, we developed a sensitive TaqMan RNA PCR method for detection of HPV16 E7 transcript in FFPE samples of HNSCC and compared its performance with another RNA PCR assay, a DNA genotyping assay and a composite method of p16 IHC and HPV DNA genotyping. We hope that the design of forthcoming treatment regimens and clinical trials of new therapies tailored for HPV positive tumors, such as HNSCC and cervical cancer, can benefit from the information provided by such a test. Further optimization and validation in larger set of clinical samples from multiple sources may be required to demonstrate the potential value of the test in clinical settings.

REFERENCES

1. C Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst. 2000; 92:709–20.

2. Posner MR, Lorch JH, Goloubeva O, Tan M, Schumaker LM, Sarlis NJ, et al. Survival and human papillomavirus in oropharynx cancer in TAX 324: a subset analysis from an international phase III trial. Ann Oncol. 2011; 22:1071-1077.

3. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tân PF, et al. Human Papilloma virus and Survival of Patients with Oropharyngeal Cancer. N Engl J Med. 2010; 363: 24-35.

4. Mirghani H, Amen F, Blanchard P, Moreau F, Guigay J, Hartl DM, et al. Treatment de-escalation in HPV-positive oropharyngeal carcinoma: Ongoing trials, critical issues and perspectives. Int J Cancer. 2015; 136: 1494-1503.

5. Seethala RR, Weinreb I, Carlson DL, McHugh JB, Harrison LB, Richardson MS, et al. Protocol for the examination of specimens from patients with carcinomas of the pharynx. 2013 Oct. Available from: http://www.cap.org/apps/docs/committees/cancer/cancer_protocols/2013/Pharynx_13protocol_3300.pdf

6. O'Sullivan B, Huang SH, Su J, Garden AS, Sturgis EM, Dahlstrom K, et al: Development and validation of a staging system for HPV-related oropharyngeal cancer by the International Collaboration on Oropharyngeal cancer Network for Staging (ICON-S): A multicentre cohort study. Lancet Oncol. 2016;17:440-451

7. Trimble CL, Frazer IH: Development of therapeutic HPV vaccines. Lancet Oncol. 2009;10:975-980

8. Doran SL, Stevanovic S, Adhikary S, Gartner JJ, Jia L, Kwong MLM, et al. T-Cell receptor gene therapy for human papillomavirus–associated epithelial cancers: A First-in-Human, Phase I/II Study. J Clin Oncol. 2019; 37:2759-2768.

9. Westra WH. Detection of human papillomavirus (HPV) in clinical samples: Evolving methods and strategies for the accurate determination of HPV status of head and neck carcinomas. Oral Oncol. 2014;50:771-779

10. Schache AG, Liloglou T, Risk JM, Filia A, Jones TM, Sheard J, et al. Evaluation of human papilloma virus diagnostic testing in oropharyngeal squamous cell carcinoma: sensitivity, specificity, and prognostic discrimination. Clin Cancer Res. 2011;17:6262-71.

11. Ukpo OC, Flanagan JJ, Ma XJ, Luo Y, Thorstad WL, Lewis JS., Jr. High-risk human papillomavirus E6/E7 mRNA detection by a novel in situ hybridization assay strongly correlates with p16 expression and patient outcomes in oropharyngeal squamous cell carcinoma. Am. J. Surg. Pathol. 2011;35:1343–1350.

12. Bishop JA, Ma XJ, Wang H, Luo Y, Illei PB, Begum S, et al. Detection of transcriptionally active high-risk HPV in patients with head and neck squamous cell carcinoma as visualized by a novel E6/E7 mRNA in situ hybridization method. Am. J. Surg. Pathol. 2012;36:1874–1882.

13. Rietbergen MM, Snijders PJF, Beekzada D, Braakhuis BJM, Brink A, Heideman DAM, et al. Molecular characterization of p16-immunopositive but HPV DNA-negative oropharyngeal carcinomas. Int J Cancer. 2014;134:2366-2372.

14. Holzinger D, Schmitt M, Dyckhoff G, Benner A, Pawlita M, Bosch FX. Viral RNA patterns and high viral load reliably define oropharynx carcinomas with active HPV16 involvement. Cancer Res. 2012; 72: 4993-5003.

15. Smeets SJ, Hesselink AT, Speel E-JM, Haesevoets A, Snijders PJF, Pawlita M, et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. Int J Cancer. 2007; 121: 2465-2472.

16. Gao G, Chernock RD, Gay HA, Thorstad WL, Zhang TR, Wang H, et al. A novel RT-PCR method for quantification of human papillomavirus transcripts in archived tissues and its application in oropharyngeal cancer prognosis. Int J Cancer. 2013; 132:882-90.

17. Lechner M, Liu J, Masterson L, Fenton TR. HPV-associated oropharyngeal cancer: epidemiology, molecular biology and clinical management. Nat Rev Clin Oncol. 2022;19:306–327.

18. Jordan RC, Lingen MW, Perez-Ordonez B, He X, Pickard R, Koluder M, et al. Validation of methods for oropharyngeal cancer HPV status determination in US cooperative group trials. Am J Surg Pathol. 2012;36:945-954.

19. Masterson L, Winder DM, Ball SLR, Vaughan K, Lehmann M, Scholtz LU, et al. Molecular analyses of unselected head and neck cancer cases demonstrates that human papillomavirus transcriptional activity is positively associated with survival and prognosis. BMC Cancer. 2016;16:367.