Application of the Roche Cobas® HPV 4800 in Formalin-Fixed, Paraffin-Embedded Samples for Head and Neck Squamous Cell Carcinomas

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Received: 24 August 2020 / Accepted: 16 October 2020 / Published online: 31 October 2020
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Abstract

Testing for high risk human papillomavirus (HR-HPV) status is standard of care in squamous cell carcinomas of the oropharynx as well as cervical lymph node squamous cell carcinomas of unknown primary origin. DNA or RNA in-situ hybridization (ISH) and p16 immunohistochemistry, widely used currently for HPV detection are operator-dependent. In addition, DNA ISH has a relatively low sensitivity, and p16 is not entirely specific for HR-HPV infection. In this study, we examined the performance of the cobas® HPV genotyping assay in formalin-fixed, paraffin-embedded (FFPE) samples of head and neck squamous cell carcinoma. FFPE samples from head neck and other anatomic sites tested by ISH and p16 for HR-HPV at ARUP Laboratories were selected for this study. Samples were deparaffinized, stained and micro-dissected for tumor contents followed by tissue lysis, then tested with cobas® for HR-HPV. All the samples were also tested by HPV Linear Array for confirmation. All (N = 18) high risk HPV positive specimens tested by cobas® were confirmed as positive by the Linear Array test. All the specimens tested as negative by cobas® were tested as negative (N = 5) or positive only for low risk HPV (N = 3) by Linear Array, as cobas® only detects HR HPV. Limits of detection for HPV16 and 18 were established at 160–320 and 320–1600 copies, respectively. Our data suggest that cobas® HR-HPV genotyping is a viable option for detection of HR-HPV in formalin-fixed, paraffin-embedded samples from head and neck and other anatomic sites and has been validated for clinical use.

Keywords Human papillomavirus · HPV · Head and neck squamous cell carcinoma · HNSCC · Cobas · Head and neck cancer

Background

Head and neck (HN) cancers account for approximately 4% of all cancers in US, and they are usually squamous cell cancers, which are referred to as squamous cell carcinomas

of the head and neck (HNSCC) [1]. About 25% of the HNSCCs are caused by high-risk human papillomavirus (HR-HPV) [2]. HPV-mediated SCCs are generally oropharyngeal (OPSCC). HPV16 is the most frequent HPV subtype detected in oropharyngeal squamous cell carcinomas (OPSCC), as this subtype is detected in 90% of the HPV-positive OPSCC. Patients with HPV-mediated OPSCCs have a better response to chemotherapy and radiotherapy than the patients with HPV negative OPSCCs [3]. As such, the determination of HPV status is standard of care for OPSCCs.

Testing for HPV status in this setting is currently done using either DNA or RNA in-situ hybridization (ISH) or p16 immunohistochemistry (IHC) on formalin-fixed, paraffin-embedded (FFPE) specimens. DNA ISH tests have a relatively low sensitivity, are highly dependent on the experience of the pathologist and are also labor intensive. p16 IHC has variable specificity for demonstrating HPV tumorigenesis

Detection of high-risk HPV plays a critical role in the management of head and neck cancer patients. This study validated the application of an automatic high-risk HPV detection system for head and neck cancers in a reference lab.

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as it is a surrogate marker of HPV infection and does not directly identify the presence of HPV [4, 5].

The cobas® HPV genotyping assay is an In Vitro Diagnostic, FDA-approved nucleic acid amplification test for the qualitative detection of HPV DNA in human cervical specimens collected in PreservCyt Solution (ThinPrep, Hologic, Marlborough, Ma, USA) using an endocervical brush/spatula. This system includes two components, the cobas® x480 for DNA extraction and the cobas® z 480 for DNA amplification and detection. The DNA of 14 High-Risk (HR) HPV targets (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) is amplified by real-time PCR, and the presence of any of these HR-HPVs is detected by a fluorescence-labeled probe. HPV16 and HPV18 are specifically genotyped using the cobas® HR HPV assay, while the other HR-HPV types are reported as “Other HR-HPV”. The cycle threshold (Ct), which is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level) is calculated for each sample. Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample). Detection of human beta globin (BG) is included in the system for monitoring the cellularity of specimens and validity of the results for each specimen [6].

This study was performed to establish the performance of the Roche cobas® HR-HPV assay for detection of HR-HPV in HNSCC FFPE specimens and to potentially validate this type of specimen for clinical testing of HR-HPV DNA in HNSCC.

Materials and Methods

Specimens

A total of 22 different squamous cell carcinomas (SCCs) from a variety of anatomic sites were included in this study. Most of the samples (N = 18) were from the head and neck, and four samples were from other anatomic sites. The study also included four cell block specimens for control purposes, which were prepared from three different HPV cell lines (HeLa for HPV18, CaSki and SiHa for HPV16) and a pleural effusion sample which was pre-tested as HPV negative. Detailed information for the case types is listed in Table 1. Patient specimens were selected by screening archived HR-HPV ISH or p16 IHC testing results performed in the clinical lab at ARUP Laboratories, Salt Lake City, UT, USA. All of the FFPE specimens were cut as five micron sections. A hematoxylin and eosin (H&E) stain and an aniline blue (AB) stain were performed on the tissue sections. The H&E stained slide was reviewed by a pathologist for tumor content. The tumor area was outlined by the reviewing

pathologist, and micro-dissected for all the downstream analysis. The use of patient specimens was approved by the Institutional Review Board of University of Utah, to which ARUP Laboratories is affiliated.

The Linear Array HPV Genotyping Test

Due to the limitations of HR-HPV ISH and p16 IHC methods mentioned above, determination of analytical accuracy for cobas® HPV was not calculated from these two tests. Instead, the Linear Array HPV Genotyping (LA) test (Roche Diagnostics, Basel, Switzerland) was used as reference test for the accuracy of cobas® HPV testing. The LA is a time-consuming, and more research lab-oriented test but can specifically genotype all the 14 h-HPV. LA utilizes amplification of target DNA by PCR and nucleic acid hybridization and has been designed to detect thirty-seven anogenital HPV DNA genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39, and CP6108). In brief, according to the manufacturer's procedure, the DNA was extracted from FFPE tissue using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). Then, the genomic DNA was amplified by PCR using primer mix containing the sequences for the above-mentioned 37 HPV genotype and the human beta-globin gene. The resultant PCR product was hybridized with DNA array strip containing the band of specific HPV DNA. The beta-globin served as internal control of DNA quality. The LA results were read according to the presence or absence of the corresponding color bands on the hybridization strip.

HR-HPV Genotyping by Cobas® 4800

cobas® 4800 genotyping is a real time PCR based technology which can simultaneously detect all 14 h-HPV types. In brief, the FFPE tumor tissue was micro-dissected from the aniline blue stained slide and transferred into an Eppendorf tube, followed by the digestion with 100 µl of QuickExtract FFPE DNA Extract reagent (Epicentre Biotechnologies, Chicago, IL, USA) for 1 h at 56 °C, and 2 min at 98 °C to inactivate the reaction. Then, the tissue lysate was mixed with 2 ml PreservCyt (Hologic, Marlborough, MA, USA), and treated same as a routine ThinPrep cervical specimen for HR-HPV genotyping using the cobas® 4800 instrument according to the manufacturer's protocol. A HPV positive FFPE tissue (positive control) and a water tube (no template control) were included for each run.

Limit of Detection

The limit of detection (LOD) for HR HPV genotypes HPV16 and HPV18 in FFPE specimens was determined using the

Table 1 Accuracy of HPV detection by cobas® 4800

No	Sample source	Diagnosis	p16 IHC result	DNA ISH result	Linear array result	cobas® result	Concordance DNA ISH /cobas®	Concordance Linear array/ cobas®
1	SiHa cell line	Cell line	NP ^a	NP	HPV16	HPV16	NP	Yes
2	Tongue	SCC	NP	HR-HPV	HPV16	HPV16	Yes	Yes
3	Nasopharynx	SCC	NP	HR-HPV	HPV16	HPV16	Yes	Yes
4	Tonsil	SCC	NP	HR-HPV	HPV16	HPV16	Yes	Yes
5	Tonsil	SCC	NP	HR-HPV	HPV16	HPV16	Yes	Yes
6	Tonsil	SCC	NP	HR-HPV	HPV16	HPV16	Yes	Yes
7	Vallecula	SCC	NP	HR-HPV	HPV16	HPV16	Yes	Yes
8	Tonsil	SCC	NP	HR-HPV	HPV16	HPV16	Yes	Yes
9	Tonsil	SCC	NP	HR-HPV	HPV16	HPV16	Yes	Yes
10	Not specified	SCC	NP	HR-HPV	HPV16	HPV16	Yes	Yes
11	Tonsil	SCC	NP	HR-HPV	HPV16	HPV16	Yes	Yes
12	Rectum	SCC	POS	NP	HPV16	HPV16	NP	Yes
13	Tongue	SCC	POS	NP	HPV16	HPV16	NP	Yes
14	Tonsil	SCC	POS	NP	HPV16	HPV16	NP	Yes
15	CaSki cell line	Cell line	NP	NP	HPV16	HPV16	NP	Yes
16	HeLa cell line	Cell line	NP	NP	HPV18	HPV18	NP	Yes
17	Tonsil	SCC	NP	HR-HPV	HPV58	POS other HR	Yes	Yes
18	Lung	SCC	NP	HR-HPV	HPV68	POS other HR	Yes	Yes
19*	Labia	SCC	NP	HR-HPV	HPV6	NEG	No	Yes
20*	Larynx	SCC	NP	HR-HPV	HPV6	NEG	No	Yes
21	Soft palate	SCC	NP	NEG	HPV11	NEG	Yes	Yes
22	Oral nodule	SCC	POS	NEG	NEG	NEG	Yes	Yes
23	Body fluid	NSCLC	NP	NP	NEG	NEG	NP	Yes
24	Vocal cord	SCC	NP	NEG	NEG	NEG	Yes	Yes
25	Tonsil	SCC	NEG	NP	NEG	NEG	NP	Yes
26	Larynx	SCC	NEG	NP	NEG	NEG	NP	Yes

*Specimens tested as positive by DNA ISH, but negative by both LA and cobas®

^aNP not performed, SCC squamous cell carcinoma, NSCLC non-small cell lung cancer

HeLa (HPV18) and SiHa (HPV16) cell lines. The cell lines were diluted in the culture medium below, above, and at the expected LOD levels (10,000, 2000, 400, 80, 16, 3.2 and 0 cells per ml), and mixed with the pleural effusion samples collected from patients with non-small cell lung carcinoma or ovarian carcinoma, which tested as HPV-negative by LA. The cell mixture was pelleted and coagulated using plasma and thromboplastin clotting method. Then, the clot was fixed in formalin and embedded in paraffin, thus undergoing the same processing (with the addition of the clotting step) as all the other surgical pathology FFPE tissues used in this study. To test the LOD, the cell block was deparaffinized using HemoDe (Fisher Scientific, Hampton, NH), followed by digestion with QuickExtract FFPE DNA Extract reagent. After mixture with PreservCyt, the samples were tested for HR-HPV genotyping in cobas® 4800 as mentioned above. Six replicates at each concentration underwent cobas® HR-HPV genotyping. The LOD was calculated based on the

lowest number of cell/mL detected in all 6 replicates. The LOD was then converted to viral copy number according to published literature [5].

Results

A total of 22 FFPE samples, including 18 HNSCC samples and four SCC samples from other anatomic sites were tested in this study (Table 1). Four HPV-positive cell lines and a HPV-negative body fluid specimen were processed into cell blocks for test control purposes. Eighteen specimens tested positive for HR-HPV by cobas®, all of which were confirmed as positive for HR-HPV by LA. Two of the positive results were shown to be types HPV58 and HPV68 by LA respectively, and were reported as positive for “Other HR-HPV” by cobas®, as cobas® only specifically genotypes the individual HPV16 and HPV18 types. All of the HPV16

and 18 positive samples had a concordant genotype result between the cobas® and LA tests. Of the 8 h-HPV negative specimens, three tested as low risk HPV positive (HPV6/11) by LA. These are not discordant results as cobas® does not report any low risk HPV types. In these cases, both testing methods reported the 3 specimens as negative for HR-HPV. Overall, all 26 specimens had a concordant result between cobas® and LA with regard to the HR-HPV status.

Interestingly, when comparing the cobas/LA results with the previous clinical testing results from DNA ISH or p16 IHC, three specimens showed discordant results. Two samples were previously tested as positive for HPV by DNA ISH, but were negative by cobas® and positive for a low risk HPV (HPV6) by LA. A third sample was positive by p16 IHC, but negative by both cobas® and LA. This sample was re-tested using HPV RNA ISH, which has higher specificity for HPV detection, and the result was negative for HR-HPV.

LOD results for HPV on FFPE specimens were obtained by using the two HPV-positive cell line cell blocks, SiHa (HPV16) and HeLa (HPV18) (Table 2). By converting the cell number to the published viral copies for each cell line (1–2 and 10–50 DNA copies for SiHa and HeLa), the LOD of cobas® test was identified as 160–320 copies of HPV16 and 320–1600 copies of HPV18 in 1 ml PreservCyt [5].

Discussion

HNSCC is a one of the major cancers affecting people worldwide, especially in developed countries. Although the overall incidence for this cancer is decreasing, the incidence of HPV positive OPSCC is actually on the rise. The HR-HPV positive cases from our current study were also mostly located in this unique anatomic site. Some authors have speculated that this anatomic site, specifically the tonsillar

crypts, may mimic the cervical transformation zone in the pathogenesis of HPV-induced squamous cell carcinoma [3].

Studies have shown that HPV positive HNSCCs behave differently when compared with the HPV negative HNSCCs. For example, HPV positive HNSCCs occurs in younger patients and in patients who are less likely to have a history of alcohol and tobacco abuse. Most importantly, HPV positive OPSCCs have better a prognosis than the HPV negative OPSCCs and HNSCCs in general because they are more sensitive to the chemotherapy and radiotherapy [7]. Based on these differences in manifestation, clinical trials have investigated, or are currently investigating, in which the HPV positive OPSCC patients are receiving so-called “de-escalation treatment.” In these trials, HPV positive patients are treated by replacing the chemotherapy with less toxic chimeric monoclonal antibodies, or by using lower radiation dose, in order to improve patient quality of life but without compromising survival [3, 8]. The detection of HPV is thus standard of care as part of the diagnostic workup for OPSCC (or level 2/3 cervical lymph node SCC of unknown primary origin) for the reasons mentioned above.

Recently, the College of American Pathologists (CAP) released an HPV testing recommendation which indicates that p16 IHC can be used as a standalone test for HR-HPV status in OPSCCs or SCCs of unknown primary origin involving level two or three cervical lymph nodes that have non-keratinizing morphology. This determination was made by evidence that p16 has robust sensitivity and specificity when it is implemented in this targeted fashion, despite the fact that it is a surrogate marker for HR HPV as it does not directly detect viral genetic material or proteins. In addition, p16 is a relatively easy and cost effective strategy from both laboratory as well as a workflow perspective [9]. On the other hand, detection of HPV mRNA, especially the E6 and E7 mRNA, is considered as the gold standard for identifying an active HR-HPV infection and for monitoring the tumorigenesis of this virus. However, the testing of mRNA frequently requires fresh and unfixed tissue, which makes it less practical for use with clinical specimens. More recently, an RNA in situ technology, RNAscope (ACD, Bio-Techne, Newark, CA, USA), was developed, which can be used to detect HPV directly from FFPE specimens [10, 11]. A recent study using the CAP proficiency testing surveys showed that HPV RNA ISH had a higher sensitivity than DNA ISH [12]. While HPV RNA ISH has been a large improvement over DNA ISH, the ISH technique still remains more subjective and operator-dependant as compared to molecular testing modalities, most due to the automation of the latter. Furthermore, both DNA and RNA ISH are still highly specialized technologies for clinical laboratories and the issue of sensitivity is a concern for at least DNA ISH. Finally, while p16 is relatively easy to adapt to the routine workflow of the clinical lab, it has imperfect specificity.

Table 2 Limit of detection levels for HPV16 and HPV18

Cells/ml	SiHa (HPV16)		HeLa (HPV18)	
	No. positive/ No. test	Mean $\pm$ SD Ct ^a	No. positive/ No. test	Mean Ct
10,000	6/6	31.4 $\pm$ 0.2	6/6	28.7 $\pm$ 0.2
2000	6/6	33.9 $\pm$ 0.2	6/6	31.4 $\pm$ 0.3
400	6/6	36.7 $\pm$ 0.4	6/6	34.2 $\pm$ 0.3
80	6/6 [#]	38.7 $\pm$ 0.4	6/6	36.5 $\pm$ 0.5
16	1/6	40.4	6/6 [#]	39.7 $\pm$ 0.3
3	0/6	NA	1/6	38.9
0	0/6	NA	0/6	NA

^aCt cycle threshold, NA not applicable

[#]The lowest concentration in which all the 6 replicates tested as positive for each cell line

HR-HPV genotyping by cobas® was FDA approved for the primary screening of cervical cancer from liquid cytology material. It is a highly automatic and well-established testing method that is widely used in many clinical labs. Some studies have looked into modifying the cobas® test for FFPE specimens [13, 14].

In the current study, we introduced a pretreatment step to digest the FFPE with a commercially available reagent for the downstream cobas® test. In so doing, we are able to incorporate the FFPE specimens with the routine cervical specimens into the same run used for cervical cytology specimens. To make the test more specific, only the tumor tissue is micro-dissected for HPV testing in our study, which comes with at least two benefits. First, it significantly decreases the possibility of a false negative call due to the amplification of BG from the benign tissues. Second, the micro-dissection of tumor tissue will also potentially avoid detecting the viral DNA from the transient HPV infection in the benign tissue. In our study, two specimens tested as positive for HR-HPV by DNA ISH, but negative by cobas®, and positive for low risk type (HPV6) by LA. It is unclear if the positive HR-HPV results were caused from a non-specific cross reactivity of the low risk HPV. A third sample tested as positive by p16 in clinical lab, but negative by both cobas® and LA. Considering the specificity issue of p16, we re-tested this sample with RNA ISH and received a negative result. These findings may be related to the known specificity issues that DNA ISH and p16 IHC tests have. Of note, in a similar study by Kerr et al., three false positive cases by cobas® were reported. Their method differed from ours as it employed a manual deparaffinization step [14]. Instead, in our method sections are stained with aniline blue, a protocol that removes the paraffin and retains stained tissue for micro-dissection. When working with PCR based testing, cross contamination is major concern for clinical laboratories, so minimizing the necessary manual pretreatment steps is a useful strategy. It is not, however, clear if the three false positive cases observed in the study by Kerr et al. were due to cross contamination.

PCR has the advantage of being fast, low cost, and highly sensitive testing for HPV detection. A major concern for HPV DNA detection by PCR is, of course, that it can target the viral DNA from transient infections, which is less biologically significant for tumorigenesis. If combined with the p16 IHC testing, the cobas® will be an even more valuable testing for HNSCC specimens. Based on the results of this study, this method underwent a full clinical validation using the cobas® platform, and has been successfully adapted into the clinical utilization with HNSCC FFPE specimens at ARUP Laboratories.

Funding None.

Compliance with Ethical Standards

Conflict of interest All authors declared that they have no conflict of interest to disclosed.

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