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Original contribution

RNA chromogenic in situ hybridization assay with clinical automated platform is a sensitive method in detecting high-risk human papillomavirus in squamous cell carcinoma☆

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Summary Detection of active human papillomavirus (HPV) is clinically important because its presence has been shown to correlate with favorable clinical outcomes and better response to treatment in oropharyngeal squamous cell carcinomas. Using a clinical automated platform, we compared the performance of commercially available HPV DNA and RNA in situ hybridization (ISH) probes in archival tissues of 57 squamous cell carcinomas. Importantly, a clinical automated platform gives (1) consistent and reproducible results for HPV ISH and (2) better standardization across clinical laboratories. Compared with polymerase chain reaction results, RNA ISH exhibited 93% concordance versus 81% of DNA ISH. RNA ISH was more sensitive than DNA ISH (100% versus 88%) and more specific (87% versus 74%). When only accounting for 2+-3+ positivity, sensitivity was 92% for RNA ISH versus 73% for DNA ISH, highlighting the ease of interpretation. p16 exhibited 96% sensitivity, whereas specificity was only 55%. In 3 cases, both RNA and DNA ISH were positive, whereas polymerase chain reaction results were negative, suggesting that ISH methods might be a more sensitive method. Performing on a clinical automated platform, RNA ISH is sensitive in determining high-risk HPV status in formalin-fixed, paraffin-embedded tissues and has the potential of being a standalone clinical test.

1. Introduction

Although more than 100 subtypes of human papillomavirus (HPV) have been reported, there are approximately 40 subtypes of HPV that can cause genital lesions, and about 15 subtypes are considered to be high risk (HR) with oncogenic potential [1]. Low-risk HPV, including most commonly types 6 and 11, are present in condyloma acuminata, which do not progress to carcinoma [2]. On the contrary, high-risk HPV, most commonly types 16 and 18, has been reported to be the etiologic agent for approximately 5% of all cancers worldwide [1]. Cervical cancers are invariably caused by high-risk HPV, with types 16 and 18 detected in 70% of cases [1]. HPV16 accounts for 85% of anal cancers and 50% of oropharyngeal cancer [1]. HPV16 and HPV18 account for 40% of vaginal, vulval, and penile cancers [1]. HPV-related carcinoma of the

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Table 1 Summary of immunohistochemical and in situ hybridization results

	RNA ISH			DNA ISH			p16		Total [a]
	Positive 1+-3+	2+-3+	Negative	Positive 1+-3+	2+-3+	Negative	Positive	Negative	
PCR positive	26 (100%)	24 (92%)	0	23 (88%)	19 (73%)	3	24 (96%)	1	26
PCR negative	4	2	27 (87%)	8	2	23 (74%)	13	16 (55%)	31

[a] Of the 26 cases positive by PCR, 25 were positive for HPV16 and 1 case for both HPV16 and HPV18. The total cases for p16 staining were 25 with positive PCR and 29 with negative PCR.

oropharynx, characterized histologically as predominantly nonkeratinizing with basaloid morphology, is associated with enhanced chemoradiation responsiveness and better overall survival and disease-specific survival in comparison to patients with HPV-negative carcinomas [3]. Therefore, HPV testing has clinical importance, not only prognostically but also in treatment planning.

Current methods available for HPV detection include serum antibody against several HPV epitopes, type-specific and broad-spectrum HPV epitopes, real-time polymerase chain reaction (PCR) to quantify viral load, DNA in situ hybridization (ISH), RNA ISH, and immunohistochemical stain. PCR-based detection of HPV *E6* oncogene expression in frozen tissue is generally regarded as the criterion standard, although rarely performed or available clinically; HPV PCR assays are sensitive methods approved by the Food and Drug Administration for the detection and typing of HPV on formalin-fixed, paraffin-embedded tissues, but visualization of viral transcripts within the tissues is not possible by this method, unlike p16 immunostain or ISH assays using either DNA or RNA probes [4]. In addition, PCR assays cannot distinguish passenger virus from clinically relevant infection. ISH methods are more specific by providing evidence of viral DNA integration into the tumor cells (active transcription).

In daily practice, p16 immunostain has been commonly used as a surrogate marker for high-risk HPV infection in the anogenital tract. Although less expensive and highly sensitive, p16 immunostain is not a specific marker for active HPV infection, and not infrequently, p16 expression has not correlated with HPV RNA ISH results [5]. In addition, there is a subset of cervical high-grade intraepithelial lesions that has been reported to be p16 negative [6].

Currently, ISH probes are commercially available for formalin-fixed, paraffin-embedded tissue. Although prior studies have shown HPV RNA ISH methods superior to HPV DNA ISH methods [7-9], these studies used either manual or automated research protocols. Using a clinical automated platform, we aim to evaluate the performance of 2 commercially available probes on formalin-fixed, paraffin-embedded tissues—Leica DNA ISH HPV probe (Leica Microsystems, Bannockburn, IL) and Advanced Cell Diagnostics RNA ISH HPV probe (Advanced Cell Diagnostics, Newark, CA) targeting HPV E6 and E7 messenger RNA (mRNA) transcript.

2. Materials and methods

The study has been approved by Massachusetts General Hospital institutional board review (2016-P-2827). Fifty-seven cases submitted for clinical testing were identified from 2015 to 2016 in the Massachusetts General Hospital pathology files. The medical records were reviewed, and age, sex, lesion site, and tumor type were recorded.

2.1. HPV polymerase chain reaction

For all cases, Roche (Indianapolis, IN) Cobas 4800 high-risk HPV analyses were performed on formalin-fixed, paraffin-embedded tissues as a clinical test. An automated platform extracted DNA and performed real-time PCR using high-risk (HR) HPV–specific and β-globin–specific complementary primer pairs for amplification and cleaved fluorescent-labeled specific oligonucleotide probes for detection. Primer pairs and probes specific for HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 were included. The results were analyzed and categorized by software as indeterminate, negative, HPV16, HPV18, or other HR-HPV for each sample.

2.2. Immunohistochemistry and ISH

Immunohistochemical and ISH studies were performed on 5-μm-thick tissue sections using a Bond 3 automated immunostainer (Leica Microsystems). The immunohistochemical and ISH studies were scored independently by J. M. and M. P. H. Discrepancy was resolved at the multiheaded microscope with another co-author (V. N.).

2.2.1. Immunohistochemistry

Primary antibody (dilution 1:4; Ventana, Tucson, AZ) against p16 and retrieval method, Leica EDTA-based pH 9.0 epitope retrieval solution for 20 minutes, were used. Diffuse staining in greater than 50% of the tumor cells was considered positive staining.

2.2.2. RNA in situ hybridization

Tissue sections were retrieved for 15 minutes with RNAscope target retrieval (Advanced Cell Diagnostics) at 95°C. They were then hybridized for 120 minutes at 42°C with a