Acta Dermatovenerol APA

Acta Dermatovenerologica
Alpina, Pannonica et Adriatica

2012;21:73-75
doi: 10.2478/v10162-012-0021-4

Comparative evaluation of the Abbott RealTi*me* High Risk HPV test and INNO-LiPA HPV Genotyping *Extra* test for detecting and identifying human papillomaviruses in archival tissue specimens of head and neck cancers

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Abstract

Introduction: The Abbott RealTi*me* is a novel real-time PCR assay designed for concurrent individual genotyping of HPV16 and HPV18 and pooled detection of 12 HPV genotypes: HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 in cervical swab specimens. In this study, the performance of RealTi*me* for detecting HPV in formalin-fixed, paraffin-embedded tissue specimens of head and neck cancers was compared to the Innogenetics INNO-LiPA assay, which allows identification of 28 HPVs, including all 14 covered by RealTi*me*.
Methods: A total of 60 FFPE tissue specimens obtained from the same number of patients with histologically confirmed cancer of the oral cavity or oropharynx were included in the study. Following DNA extraction using a Qiagen DNA Mini Kit, RealTi*me* and INNO-LiPA were performed on all samples, as instructed by the manufacturers.
Results: A 136-bp fragment of human beta-globin serving as an internal control in RealTime was successfully amplified from all 60 tissue samples included in the study. RealTi*me* and INNO-LiPA showed 100% agreement and detected HPV DNA in 5/60 (8.3%) of the cancer samples, which all contained genotype HPV16.
Conclusions: RealTi*me* assay is a reliable, sensitive, and specific diagnostic tool for the detection and partial genotyping of targeted HPV genotypes in FFPE tissue specimens of oral and oropharyngeal cancer.

Received: 15 January 2013 | **Returned for modification:** 25 January 2013 | **Accepted:** 8 February 2013

Introduction

It has been well established that persistent infection with high-risk human papillomaviruses (HPV), the most important of which are HPV16 and HPV18, plays the leading etiological role in the development of cervical and anal cancer and their immediate precursors, and a substantial proportion of vaginal, penile, and vulvar cancers (1). In addition, recent data suggest that HPV infection, particularly with HPV16, is also etiologically linked with a subset of head and neck cancers, especially with squamous cell carcinoma of the oropharynx and, to a lesser extent, squamous cell carcinoma of the oral cavity and larynx (2-4). The presence of HPV in head and neck cancers is consistently associated with better disease prognosis; even though the HPV status of a cancer has important clinical implications, it is still not used for therapeutic decision-making outside the context of clinical trials (5).

In addition to swab specimens and fresh or fresh-frozen tissue biopsies, which are most frequently used in HPV diagnostics, tissues fixed with formalin and subsequently embedded in paraffin or paraplast (FFPE) can also be used for detecting and identifying HPV. However, sensitive and accurate detection of HPV in these specimens is particularly challenging because DNA is often fragmented due to procedural influences (e.g., excessive tissue fixation, DNA extraction) and/or environmental influences (e.g., elevated storage temperature) (6-8). Because DNA fragmentation can significantly reduce the efficiency of PCR, it is recommended to use HPV methods that amplify a relatively small portion of the viral genome (< 160-bp) when working with archival specimens. The most frequently used methods for detecting HPV in FFPE tissue specimens have been those based on SPF10 and GP5+/6+ primers, targeting 65-bp and 150-bp fragments of the HPV L1 gene, respectively (9).

The Abbott RealTi*me* High Risk HPV test (RealTi*me*; Abbott Molecular, Des Plaines, IL) is a fully clinically validated test that allows concurrent individual genotyping of HPV16 and HPV18 and pooled detection of 12 HPV genotypes: HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 (10–11). RealTi*me* is performed on a real-time PCR *m2000rt* instrument (Abbott Molecular) using a modified GP5+/6+ primer mix; co-amplification of a 136-bp fragment of human beta-globin gene is used as an internal process control for sample adequacy, DNA extraction, and amplification. The extraction of DNA can be performed manually or alternatively with a fully automated high-throughput *m2000sp* instrument or a smaller *m24sp* instrument; all three sample preparation methods are comparable in terms of DNA yield, and they use the same reagents and the same extraction procedure (11). RealTi*me* is originally validated for use with cervical swab specimens collected in various transport media (10, 12). However, it has recently been shown that DNA extracted from pre-lysed FFPE tissue specimens of cervical and head and neck cancers with the Abbot DNA extraction systems can also reliably be used for RealTi*me* (9, 13, 14).

This study evaluated the performance of RealTi*me* in comparison to the INNO-LiPA HPV Genotyping *Extra* test (INNO-LiPA; Innogenetics NV, Ghent, Belgium) for detecting and identifying HPV in 60 archival FFPE tissue specimens of head and neck cancers. INNO-LiPA is a widely used HPV genotyping test based on hybridization technology, which allows the identification of 28 different HPV genotypes, including all 14 HPVs covered by RealTi*me*. The test enables amplification of a 65-bp fragment of the HPV L1 gene using biotinylated SPF10 primers, followed by genotyping of the

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resulting amplicons with a single-typing strip containing HPV-specific oligonucleotide probes (15). To the best of our knowledge, this study is the first to comparatively evaluate RealTi*me*- and INNO-LiPA in archival tissue specimens of head and neck cancers.

Materials and Methods

A total of 60 FFPE tissue specimens obtained from the same number of Slovenian patients with histologically confirmed cancer of the oral cavity or oropharynx were included in the study. Forty-seven patients had squamous cell carcinoma and 13 patients had verrucous carcinoma, an uncommon variant of squamous cell carcinoma. Three 10 µm thick sections were cut from each tissue block and processed for DNA extraction; before sectioning, two to three outer sections were discharged. In order to prevent possible sample-to-sample contamination, both the microtome blade and working surface were cleaned of tissue and/or paraffin parts and decontaminated using DNA Away solution (Molecular Bio-Products, San Diego, CA) after each use.

DNA was extracted from the tissue sections using a DNA Mini Kit (Qiagen, Hilden, Germany), following our in-house protocol. Briefly, tissue sections were incubated with 180 µl of buffer ATL and 20 µl of proteinase K overnight, on a rocking platform at 56 °C and 400 rpm, followed by incubation at 90 °C for 1 h. In the next step, 400 µl of premixed Buffer AL and ethanol (ratio 1:1) was added; samples were mixed vigorously and transferred into QIAamp Mini spin columns. Centrifugation for 1 minute at 8,000 rpm was followed by washing the spin column membrane with 500 µl of Buffer AW1 and 500 µl of Buffer AW2. DNA was eluted with 100 µl of Buffer AE, quantified using a NanoDrop ND-2000c instrument (NanoDrop Technologies, Oxfordshire, UK), and stored at –20 °C until use.

RealTi*me* and INNO-LiPA were performed on all 60 samples, following the manufacturers' instructions. Up to 200 ng (up to 10 µl) of DNA of each sample was used per 50 µl PCR reaction because it has been previously shown that this concentration supports the most efficient PCR (16). To control for possible amplicon carryover contamination, water blanks were placed after every seventh reaction tube in all PCR runs. Samples were considered RealTi*me*-positive when the cycle threshold (Ct) value for any of the HPV genotype and beta-globin detected was less than or equal to the cutoff value of 35.

Results

A 136-bp fragment of beta-globin, serving as an internal control, was successfully amplified from all 60 tissue samples included in the study; the RealTi*me* Ct values ranged from 24.2 to 29.8 (mean 27.4; median 27.8). HPV DNA was detected using RealTi*me* in 5/60 (8.3%) of the cancer specimens; the Ct values ranged from 20.6 to 30.1 (mean 24.7; median 23.2). All RealTi*me* HPV positive samples were histologically confirmed as squamous cell carcinomas and all contained HPV16. Using INNO-LiPA, HPV DNA (HPV16) was detected in all five cancers previously positive with RealTi*me*; all other specimens tested HPV DNA–negative. All water blanks used to control for PCR contaminations were negative for internal control with RealTi*me* and HPV DNA with both tests.

Discussion

Formalin-fixation and subsequent paraffin embedding is a standard procedure for long-term preservation of tissue specimens in pathology departments worldwide. FFPE specimens, which are routinely used for histopathological purposes, can also be used for etiological and epidemiological studies of HPV and other viral infections in cases in which fresh or frozen tissue is not available. However, sensitive and accurate detection of viruses in such archival specimens may be affected because viral DNA is often degraded as a result of excessive fixation or long and/or inappropriate storage conditions. Such damage to DNA includes chemical modification, cross-linking, and fragmentation (7, 8). Therefore, HPV methods, including INNO-LiPA and GP5+/GP6+-based assays, that target small portions of viral genome are the most suitable and have been most frequently used for detecting HPV in archival tissue specimens (6, 9, 17, 18).

RealTi*me* is intended to be used with cervical swab specimens collected in various transport media (11, 12) and has been clinically validated for primary cervical cancer screening in women 30 years and older, and ASC-US triage (10). However, it has recently been shown that RealTi*me* combined with the Abbott DNA extraction system can also be used for HPV detection in FFPE tissue specimens of head and neck cancers; specifically, oral, oropharyngeal, and laryngeal cancer (13, 14). Similarly, we recently showed that RealTi*me* in conjunction with *m2000sp* is an excellent alternative to INNO-LiPA for reliable detection of 14 targeted HPV genotypes in FFPE cervical cancer tissue specimens; there was 100% genotyping agreement between the two methods on 62 samples tested for HPV genotypes that can be identified by both assays (9).

In this study, comparative evaluation of RealTi*me* and INNO-LiPA was conducted for the first time on DNA extracted from 60 FFPE tissue specimens of head and neck cancers using a Qiagen DNA Mini Kit. In order to minimize the fragmentation of DNA during isolation, an incubation step at 90 °C was introduced in order to partially reverse formalin cross-linking between nucleic acids, between nucleic acids and proteins, and between proteins, as suggested by the manufacturer (19). Both HPV assays evaluated showed equivalent performance and 100% agreement in detection of HPV DNA in 60 cancers. Successful and early (Ct < 30) amplification of the RealTi*me* internal control suggested that the recovery of amplifiable DNA from all FFPE specimens was excellent and that the extracted DNA was of adequate quality. Although INNO-LiPA co-amplifies a fragment of the human HLA-DP1 gene, which serves as internal amplification control, the result was often negative, most probably due to the relatively large target amplicon (270-bp). Because the detection of HPV-DNA by PCR in cancer tissue may not be directly linked to a causal relationship in the case of head and neck cancers (5), further evaluations are needed to see whether RealTi*me* and INNO-LiPA detect only the biologically relevant HPV infections.

The majority of previously published studies have used various organic compounds to remove paraffin before tissue lysis. However, no data indicate that paraffin disturbs tissue lysis as long as it is melted during the proteinase K incubation step (8). Although some authors believe that unremoved paraffin can lead to inhibition during subsequent PCR (20), this was not observed in our study. In addition, the deparafinization step is generally time

consuming, is associated with increased risk of sample-to-sample contamination, and can result in great tissue loss during removal of the melted paraffin and subsequent washing of the tissue with ethanol, especially when the tissue sections are small.

In conclusion, this study showed that the RealTi*me* assay in combination with the Qiagen DNA Mini Kit is a reliable, sensitive, and specific diagnostic tool for detecting and partial genotyping of targeted HPV genotypes in FFPE tissue specimens of oral and oropharyngeal cancer.

Disclosures

Abbott Molecular provided financial support for their assay used in this study. Abbott Molecular was not involved in the study design, data collection, data analysis and interpretation, or writing the manuscript. Mario Poljak has received travel grants and honoraria from Abbott Molecular for speaking and for participation at scientific conferences and sitting on the advisory board. Boštjan J. Kocjan has received a consultation fee from Abbott Molecular.

Abbott Molecular was not involved in the study design, data collection, data analysis and interpretation, or writing the manuscript.

References

1. zur Hausen H. Papillomaviruses in the causation of human cancers—a brief historical account. Virology. 2009;384:260-5.
2. Kansky AA, Poljak M, Seme K, Kocjan BJ, Gale N, Luzar B, et al. Human papillomavirus DNA in oral squamous cell carcinomas and normal oral mucosa. Acta Virol. 2003;47:11-6.
3. Kreimer AR, Clifford GM, Boyle P, Franceschi S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: A systematic review. Cancer Epidem Biomar. 2005;14:467-75.
4. Syrjänen S, Lodi G, von Bültzingslöwen, Aliko A, Arduino P, Campisi G, et al. Human papillomavirus in oral carcinoma and oral potentially malignant disorders: a systematic review. Oral Dis. 2011;17:58-72.
5. Gillison ML, Alemany L, Snijders PJ, Chaturvedi A, Steinberg BM, Schwartz S, et al. Human papillomavirus and diseases of the upper airway: head and neck cancer and respiratory papillomatosis. Vaccine. 2012;30:Suppl 5:F34-54.
6. Poljak M, Cuzick J, Kocjan BJ, Iftner T, Dillner J, Arbyn M. Nucleic acid tests for the detection of alpha human papillomaviruses. Vaccine. 2012;30:Suppl 5:F100-6.
7. Tan SE, Garland SM, Rumbold AR, Tabrizi SN. Human papillomavirus genotyping using archival vulvar dysplastic or neoplastic biopsy tissues: comparison between the INNO-LiPA and linear array assays. J Clin Microbiol. 2010;48:1458-60.
8. Gilbert MT, Haselkorn T, Bunce M, Sanchez JJ, Lucas SB, Jewell LD, et al. The isolation of nucleic acids from fixed, paraffin embedded tissues—which methods are useful when? PLoS One. 2007;2:e537.
9. Kocjan BJ, Seme K, Poljak M. Comparison of the Abbott RealTi*me* High Risk HPV test and INNO-LiPA HPV Genotyping *Extra* test for the detection of human papillomaviruses in formalin-fixed, paraffin embedded cervical cancer specimens. J Virol Methods. 2011;175:117-9.
10. Poljak M, Oštrbenk A, Seme K, Učakar V, Hillemanns P, Vrtačnik Bokal E, et al. Comparison of clinical and analytical performance of the Abbott RealTi*me* High Risk HPV test and Hybrid Capture 2 in population-based cervical cancer screening. J Clin Microbiol. 2011;49:1721-9.
11. Huang S, Tang N, Mak WB, Erickson B, Salituro J, Li Y, et al. Principles and analytical performance of Abbott RealTi*me* High Risk HPV test. J Clin Virol. 2009;45:Suppl 1:S13-7.
12. Poljak M, Kovanda A, Kocjan BJ, Seme K, Jančar N, Vrtačnik-Bokal E. The Abbott RealTi*me* High Risk HPV test: comparative evaluation of analytical specificity and clinical sensitivity for cervical carcinoma and CIN 3 lesions with the Hybrid Capture 2 HPV DNA test. Acta Dermatovenerol Alp Panonica Adriat. 2009;18:94-103.
13. Snietura M, Jaworska M, Pigłowski W, Goraj-Zając A, Woźniak G, Lange D. High-risk HPV DNA status and p16 (INK4a) expression as prognostic markers in patients with squamous cell cancer of oral cavity and oropharynx. Pol J Pathol. 2010;61:133-9.
14. Snietura M, Piglowski W, Jaworska M, Mucha-Malecka A, Wozniak G, Lange D, et al. Impact of HPV infection on the clinical outcome of p-CAIR trial in head and neck cancer. Eur Arch Otorhinolaryngol. 2011;268:721-6.
15. Poljak M, Kocjan BJ. Commercially available assays for multiplex detection of alpha human papillomaviruses. Expert Rev Anti Infect Ther. 2010;8:1139-62.
16. Evans MF, Adamson CS, Simmons-Arnold L, Cooper K. Touchdown General Primer (GP5+/GP6+) PCR and optimized sample DNA concentration support the sensitive detection of human papillomavirus. BMC Clin Pathol. 2005;5:10.
17. Rubin MA, Kleter B, Zhou M, Ayala G, Cubilla AL, Quint WG, et al. Detection and typing of human papillomavirus DNA in penile carcinoma: evidence for multiple independent pathways of penile carcinogenesis. Am J Pathol. 2001;159:1211-8.
18. Odida M, de Sanjose S, Sandin S, Quiros B, Alemany L, Lloveras B, et al. Comparison of human papillomavirus detection between freshly frozen tissue and paraffin embedded tissue of invasive cervical cancer. Infect Agent Cancer. 2010;5:15.
19. Unlocking your FFPE archive: critical factors for molecular analysis of FFPE samples [internet]. QIAGEN; 2013 [cited 2013 Jan 15]. Available from: http://www.qiagen.com/literature/render.aspx?id=200780.
20. Stanta G, Schneider C. RNA extracted from paraffin embedded human tissues is amenable to analysis by PCR amplification. Biotechniques. 1991;11:304-8.