World Cancer Research Journal WCRJ 2015; 2 (1): e497

DEFINING THE BETTER ALGORITHM FOR THE ACCURATE IDENTIFICATION OF HPV STATUS AMONG OROPHARYNGEAL SQUAMOUS-CELL CARCINOMA. RESULTS FROM A PILOT STUDY

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Abstract: **Background:** *The recognition of tumor infection by human papilloma virus (HPV) in oropharyngeal squamous-cell carcinoma (OSCC) is emerging as a valid biomarker to more accurate selection of patients for specific treatment, surveillance and tumor staging. To this aim, the HPV detection strategy in OSCC must dissect between HPV that is acting as a driver of malignant transformation, and transcriptionally silent virus involved in productive infection. The aim of this study is to define the better method for the accurate identification of HPV status among OSCC.*

Patients and Methods: *Thirty-six patients were selected for HPV status assessment combining different methods, such as immunohistochemistry (IHC) for p16, in-situ hybridization (ISH) for high risk (HR)-HPV DNA and HR-HPV E6/E7 mRNA along with real-time PCR of HPV16 E6/E7 mRNA. All these cases were originally classified as HPV negative by DNA-based ISH but p16 positive by the IHC.*

Results: *Twenty-six cases showed concordance between methods; whereas, nine cases resulted negative for HPV E6/E7 mRNA RT-PCR but positive for HPV E6/E7 mRNA ISH.*

Conclusion: *By considering that the bright field HPV E6/E7 mRNA ISH could be more sensitive than mRNA-based real-time RT-PCR, and that it provides the precise identification of transcriptionally active HPV infected cells, a randomized analysis to validate the robustness of this preliminary assay will be undertaken.*

Key words: *HPV status, Oropharyngeal squamous-cell carcinoma, OSCC, Algorithm, HPV viral infection.*

INTRODUCTION

Background and rationale: There is growing body of evidence that high risk papillomavirus (HR-HPV)-positive oropharyngeal squamous-cell carcinoma (OSCC) differs significantly from HPV-negative OSCC in terms of risk-factor profiles, molecular characteristics, and clinical-pathologic features. Notably, patients with HPV-positive OSCC have a better prognosis compared to those with HPV-negative OSCC and most likely benefit from specific treatments[1-6]. The recognition of tumor infection by HPV in OSCC is emerging as a valid biomarker to more accurate selection of patients for specific treatment, surveillance and tumor staging (e.g. localization of tumor origin). To this, the final goal of any HPV detection strategy in OSCC, is not only the identification of HPV, but to dissect between HPV that is acting as a driver of malignant transformation, and transcriptionally silent virus involved in productive infection (i.e. passenger virus).

Defining the better algorithm for the accurate identification of HPV status among OSCC: Various techniques with different sensitivity and specificity are currently in use for HPV detection, ranging from consensus and type-specific PCR methods, real-time PCR assays, DNA in-situ hybridization (ISH), HPV

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E6/E7 mRNA ISH[7], and immunohistochemical detection of surrogate biomarkers (e.g., P16 protein) (Table 1). In this regards, p16 immunohistochemistry (IHC) alone has been advocated as the best test to use for risk stratification in OSCC[8]. However, none of the single methods offers optimal sensitivity and specificity levels. Therefore, stepwise algorithms that combine different HPV tests have been proposed as a strategy to compensate for the limitations of individual tests. We designed an algorithm which uses multimodality methods of HPV detection beginning with HR-HPV DNA ISH and p16 IHC methods[9] (Figure 1 A).

HR-HPV DNA ISH allows the identification of infected cells and provides information regarding HPV's causal role as it discriminates between episomal state linked to infective process marked by a diffuse nuclear and cytoplasmic staining and integrated state playing a pivotal role in tumorigenesis and tumor maintenance characterized by a punctuate nuclear staining (Figure 2). These two different HPV footprint signatures are also named "infection patterns". A further tool to identify HPV causative infection is the IHC staining for p16. In fact, in HPV DNA positive cases p16 overexpression is an indirect marker of active HPV E7 oncogene transcription[10,11]. By combining the results from HR-HPV DNA ISH and p16 IHC we are able to sort out true HPV positive cases (namely HR-HPV DNA positive/p16 positive) from HPV negative cases (namely HR-HPV DNA negative/p16 negative). In cases expressing p16 without evidence of HPV infection based on DNA-ISH, mRNA-based real time RT-PCR is applied to definitively ascertain the HPV status (Figure 1 A).

Aim of the study: Refining the algorithm of HPV detection in order to make it more accurate and reproducible by comparing the sensitivity of the mRNA-based real time RT-PCR with the mRNA bright field ISH assay in detecting transcriptionally active high risk (HR)-HPV (Figure 1).

Analysis of viral infection

Methods

DNA-based in situ hybridization: DNA-based ISH has been performed using both the Inform HPV III family probe (Ventana Roche), a cocktail of probes

Table 1. Currently available HPV detection methods on formalin fixed paraffin-embedded material.

Detection technique	Advantages
Consensus HPV PCR – end point PCR (qualitative)	High sensitivity Targets numerous oncogenic strains Available commercial kit with CE certification
HPV-type specific real time PCR (quantitative)	High sensitivity High specificity Provides quantitative measure of viral load
HPV E6/E7 mRNA real time RT-PCR	High sensitivity High specificity Detects clinically relevant infections with evidence of active oncogene transcription Considered as the "gold" standard
HR-HPV DNA in situ hybridization	High specificityProvides visual detection of infected cells (preservation of tissue context) Distinction between episomal and integrated HPV Available commercial kit with CE certification Simple ad accessible to most laboratories
HR-HPV E6/E7 mRNA in situ hybridization	High sensitivity High specificity Detects clinically relevant infections with evidence of active oncogene transcription Provides visual detection of infected cells (preservation of tissue context)
P16 immunostaining	High sensitivity Provides proof of transcriptional activity Available commercial kit with CE certification Simple ad accessible to most laboratories Cost effective and adequate for routine screening in most laboratories

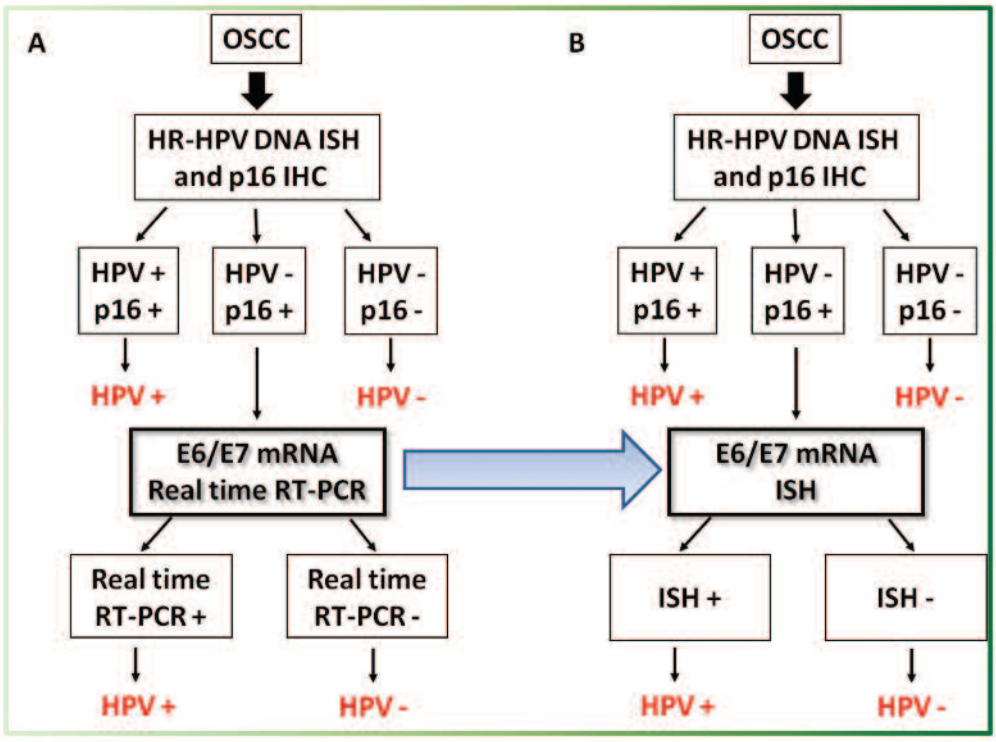

Figure 1. Multimodality detection strategies look to utilize the strengths of individual assays in combination to optimize the overall reliability of HPV detection. The algorithm proposed uses multimodality methods of HPV detection beginning with p16 immunohistochemical staining and HR-HPV in situ hybridization methods. E6/E7 mRNA based methods (either real time RT-PCR or ISH) assays are used in p16 positive/HR-HPV DNA negative carcinomas to confirm the presence of HPV.

recognizing HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 70. DNA-ISH has been performed on an automated immunostainer (BenchMark Ultra, Ventana Roche) according to the company's instructions. For each case the percentage of pattern of infection (integrated versus episomal) has been recorded (see Figure 2 for a representative example).

P16: P16 immunoreactivity will be evaluated using the CIN tec p16INK4a Histology Kit (Roche Diagnostics). A tumor has been considered positive in the case of strong nuclear and cytoplasm decoration in >50% of tumor cells. Immunohistochemistry has been performed on an automated immunostainer (BenchMark Ultra, Ventana Roche) according to the company's instructions.

HPV E6/E7 mRNA based real time quantitative RT-PCR: Real time quantitative RT-PCR assays has been performed to detect HPV16 E6/E7 mRNA as previously described[4,9].

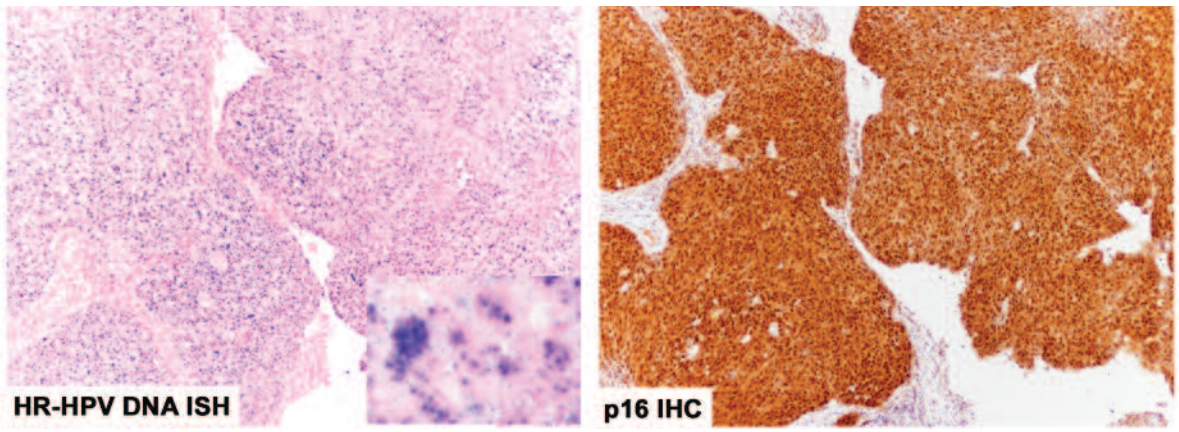

Figure 2. The presence of HPV in squamous cell carcinoma of the tonsil is documented by p16 immunohistochemistry and HPV DNA in situ hybridization.

HPV E6/E7 mRNA-based in situ hybridization: ISH for HR-HPV E6/E7 mRNA has been performed by hand using the RNAscope HPV kit (Advanced Cell Diagnostics, Inc., Hayward, CA) according to the manufacturer's instructions. In brief, 5 μm formalin fixed, paraffin-embedded tissue sections have been pretreated with heat and protease before hybridization with target probes to the HR-HPV genotypes. Probes for the following targets have been used: E6/E7 mRNA of HPV 16 alone and a cocktail of HPV E6/E7 mRNA probes recognizing 18 HR-HPV subtypes [16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82]. A horseradish peroxidase-based signal amplification system has been then bound to the target probes followed by color development with diaminobenzidine. Positive staining has been identified as brown, punctate dots present in the nucleus and/or cytoplasm (see Figure 3 for a representative example). Control probes for the bacterial gene 4-hydroxy-tetrahydrodipicolinate reductase (DapB), negative control, and for the housekeeping gene ubiquitin C (UbC), positive control for the evidence of preserved RNA, have been also included on each case.

Positive controls: Cell blocks obtained from cell lines with known copy number of HPV genomes have been used as positive control: CaSki cells (HPV16, 200-600 copies/cell), HeLa cells (HPV18, 10-50 copies/cell), SiHa cells (HPV16, 1-2 copies/cell).

Patients

Thirty-six patients belonging to a subset out of 694 retrospective OSCC cases analyzed for HPV infection and classified as HPV negative by DNA-based ISH but p16 positive by the IHC were enrolled in this pilot study. The series consisted of 31 males and 5 females, with an age ranging from 21 to 95 years (media 58).

RESULTS AND DISCUSSION

As above mentioned, HPV DNA ISH seems to be more sensitive than HPV E6/E7 mRNA RT-PCR[9]. Other studies have pointed out that HPV E6/E7 mRNA ISH is more sensitive than HPV DNA ISH[7], but no studies have directly compared the HPV E6/E7 mRNA RT-PCR versus the novel bright field ISH assay to detect HR-HPV E6/E7 mRNA.

To overcome this critical issue and to elucidated the role of mRNA-based ISH assay, we performed a pilot study focusing on a subset of 36 retrospective OSCC cases classified as HPV negative by DNA-based ISH but p16 positive by the IHC. Twenty-six cases showed concordance between methods, whereas 9 cases resulted negative for HPV E6/E7 mRNA RT-PCR but positive for HPV E6/E7 mRNA ISH and 1 case resulted positive for HPV E6/E7 mRNA RT-PCR but negative for HPV E6/E7 mRNA ISH. To confirm these preliminary data we planned to perform a randomized study that foresees the comparative analysis of 40 OSCC retrospective cases among those collected during the time window 2000-2013 and encompassing HPV DNA +/p16+, HPV DNA -/p16- and HPV DNA-/P16+ subsets. These cases, for which it is already available the DNA-ISH and p16-IHC evaluation, will be evaluated for HPV E6/E7 mRNA RT-PCR and HPV E6/E7 mRNA ISH. Each case will be blindly and independently evaluated for HPV E6/E7 mRNA ISH and HPV E6/E7 mRNA RT-PCR according to a randomized list. All the evaluations will be recorded in a dedicated database for the subsequent statistical analysis. As the classification criteria adopted involves a classification criteria on a dichotomous scale, the concordance pattern between mRNA ISH and mRNA RT-PCR will be evaluated by adopting proper indexes of concordance (Choen kappa statistics)[12,13].

PERSPECTIVES

Currently, in the cases expressing p16 without evidence of HPV infection (based on DNA-ISH), mRNA-based real time RT-PCR is applied (Figure 1A) to definitively confirm the presence of HPV active transcription footprint. By considering that the bright field HPV E6/E7 mRNA ISH could be more sensitive than mRNA-based real-time RT-PCR, and that it provides the precise identification of transcriptionally active HPV infected cells, we undertook a randomized analysis to validate the robustness of our preliminary assay. The analysis is ongoing and, if the results will be confirmed, a new algorithm will be proposed (Figure 1B). The new algorithm, which combines the DNA-based ISH and HPV E6/E7 mRNA ISH couplet with p16 expression, will be tested on series of prospective OSCC cases.

Acknowledgement. Supported in part by an Institutional Grant from Institutional grant from the Fondazione IRCSS Istituto Nazionale Tumori Milano "Validation of a new algorithm for HPV status assessment in head and neck carcinoma" (A.G).

Conflict of Interests: The Authors declare that they have no conflict of interests.

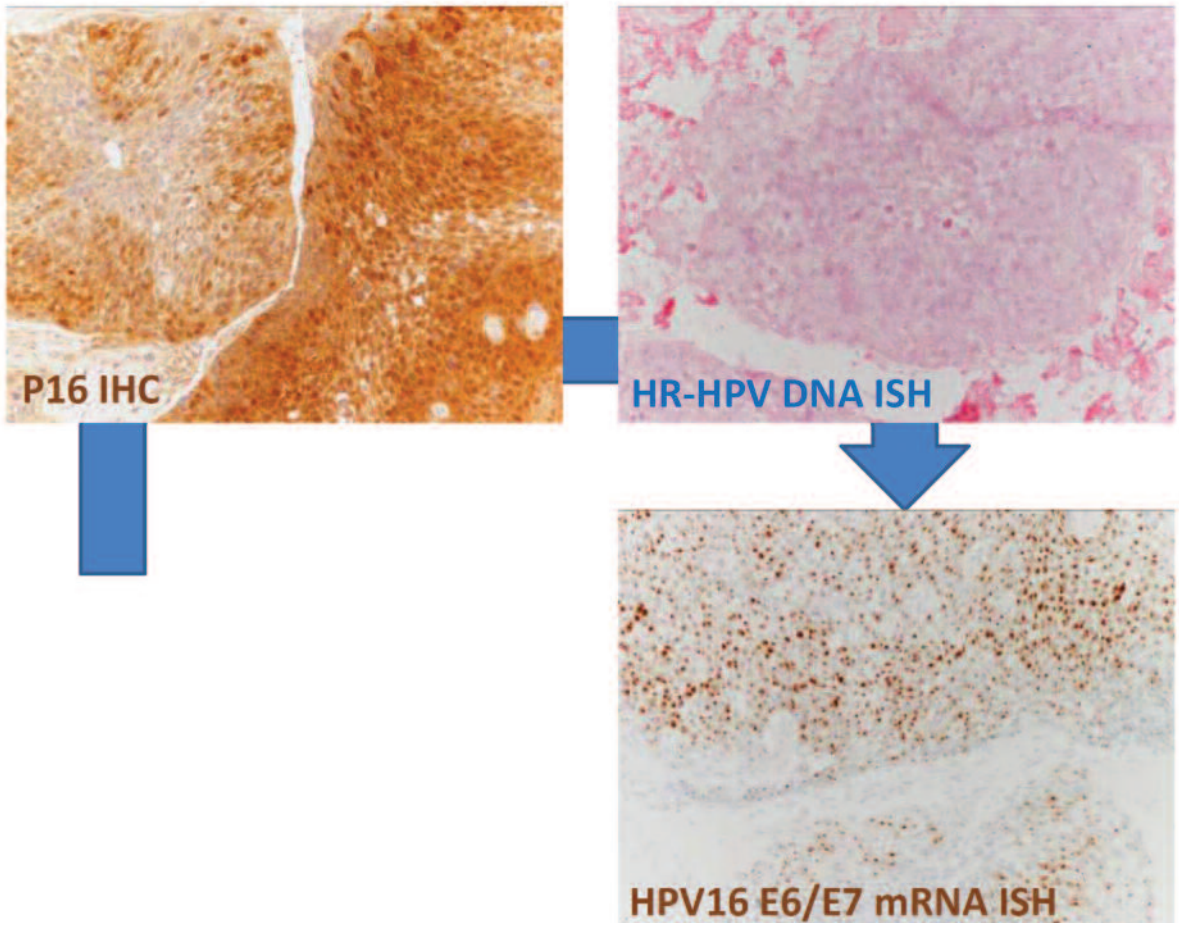

Figure 3. This HPV-related oropharyngeal carcinoma is strongly p16 positive by immunohistochemistry, HR-HPV DNA negative by in situ hybridization (ISH), and HPV E6/E7 mRNA positive by ISH.

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