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Original contribution

Detection of human papillomavirus in non–small cell carcinoma of the lung ☆

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Summary High-risk human papillomavirus (hrHPV) is an etiologic agent in squamous cell carcinoma (SqCC) arising in the oropharynx and cervix, and a proven prognostic factor in oropharyngeal SqCC. Many studies have found HPV in non–small cell lung carcinoma (NSCLC). Recent studies advocate the detection of messenger RNA transcripts of E6/E7 as more reliable evidence of transcriptively active HPV in tumor cells. The clinical significance of finding HPV remains unclear in NSCLC. This study sought to determine the prevalence of biologically active HPV infection in NSCLC comparing different methodologies. Surgical pathology material from resected primary lung adenocarcinoma (ADC; n = 100) and SqCC (n = 96) were retrieved to construct tissue microarrays. In situ hybridization (ISH) for hrHPV DNA (DNA-ISH), hrHPV E6/E7 RNA (RNA-ISH), and p16 immunohistochemistry were performed. Cases of oropharyngeal SqCC with known HPV infection were used as positive controls. Expression of p16 was scored as positive if at least 70% of tumor cells showed diffuse and strong nuclear and cytoplasmic staining. Punctate nuclear hybridization signals by DNA-ISH in the malignant cells defined an HPV-positive carcinoma. Of the 196 patients (range, 33-87 years; 108 men), p16 was positive in 19 ADCs and 9 SqCCs, but HPV DNA-ISH and RNA-ISH were negative in all cases. Our study did not detect HPV infection by DNA-ISH or RNA-ISH in any cases of primary NSCLC despite positive p16 expression in a portion of ADC and SqCC. p16 should therefore not be used as a surrogate marker for HPV infection in NSCLC.

1. Introduction

High-risk human papillomavirus (hrHPV) is considered an etiologic agent in carcinomas arising in various anatomic sites such as squamous cell carcinoma (SqCC) in the cervix [1] and oropharynx [2,3] and adenocarcinoma (ADC) in the endocervix [1]. It is also a proven prognostic factor in oropharyngeal SqCC [2]. One of the earliest reports suggesting the potential carcinogenic role of HPV in SqCC arising in bronchial epithelium dates back more than 30 years [4,5]. Since then, many studies have detected HPV in non–small cell carcinomas (NSCLC) of the lung, ranging widely from 0 to 78% [6]. Although past studies have mainly focused on SqCC, relatively high rates of HPV infection have also been found in pulmonary ADC. The

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range of HPV infection detected in lung ADC occurring in Asian populations varies from 0 to around 40% [7–9]. In contrast, several studies based on North American and European populations did not find an association between HPV infection and NSCLC [10–13]. A recent comprehensive meta-analysis [6] and an international pooled analysis [14] found that the variation in HPV detection rate may be a function of the geographical origin and histologic subtypes, rather than detection methods.

Nonetheless, the potential contribution of differences in methodology to the widely differing results between studies should be considered. Most commonly used methods to detect HPV DNA include polymerase chain reaction (PCR), Hybrid Capture II, Digene, Gaithersburg, MD and in situ hybridization (ISH). However, the sole finding of HPV DNA is not sufficient to prove a carcinogenic role in the development of NSCLC. To this purpose, demonstrating the presence of transcriptively active HPV E6 and E7 viral oncogenes is more informative. Although reverse transcription PCR (RT-PCR) is currently the preferred method to detect messenger RNA (mRNA) transcripts of these oncogenes, isolating high-quality mRNA from formalin-fixed, paraffin-embedded (FFPE) tissue is challenging and access to frozen tissue is often limited. Moreover, PCR assays do not allow using light microscopy to determine where HPV DNA and RNA are found—that is, in benign versus malignant cell populations. To overcome this difficulty, a novel method using ISH to visualize E6/E7 mRNA transcripts in tissues has been developed (RNAscope; Advanced Cell Diagnostics, Hayward, CA). A recent study comparing HPV DNA-ISH and RNA-ISH assay in head and neck SqCC found a high concordance rate between the 2 methods [15]. A significant advantage of ISH over PCR is the direct visualization of viral transcripts in tumor cells using light microscopy.

Immunohistochemistry (IHC) to detect $p16^{INK4a}$ protein is often used as a surrogate marker for HPV infection. *P16*^{INK4a} encodes for a cyclin-dependent kinase inhibitor that suppresses cell proliferation by maintaining retinoblastoma (RB) in a hypophosphorylated state [16]. In cells infected with HPV, the viral oncoprotein HPV E7 binds to and inactivates RB, consequently impeding the tumor suppressor effect of $p16^{INK4a}$. Although this leads to up-regulation—and overexpression on IHC—of *p16*^{INK4a} gene in the infected cells, the resulting high levels of $p16^{INK4a}$ do not reverse the downstream inhibitory effect of HPV E7 on RB [16]. In conditions such as SqCC and ADC of the uterine cervix, and in a portion of oropharygeal SqCC, overexpression of $p16^{INK4a}$ is strongly correlated with HPV infection and IHC for p16 is commonly used as a surrogate marker for this purpose [17]. However, this correlation is not well established in lung NSCLC [13].

The objective of this study is to determine the prevalence of HPV infection in NSCLC (ADC and SqCC) and its clinical significance in a large institution using a panel of recommended and novel detection methods.

2. Materials and methods

2.1. Case selection

This study was approved by the Mayo Clinic institutional review board, and institutional guidelines regarding research using archival human tissue were followed. Consecutive cases from 200 patients who have undergone complete surgical resection of pulmonary ADC (n = 100) or SqCC (n = 100) of the lung between 2009 and 2012 were retrieved from the surgical pathology archives of Mayo Clinic, Rochester, MN. Surgical resection was considered the primary treatment modality for all cases, with or without adjuvant therapy. Clinical and radiology records were reviewed. Histology slides from each case were reviewed for classification, grading, and pathologic staging. Four cases of SqCC were excluded because these patients had a history of SqCC of the head and neck, and therefore, a metastasis could not be entirely excluded.

2.2. Tissue microarray construction

The most representative hematoxylin and eosin (H&E) slide from each case and its corresponding FFPE tissue block was selected to harvest tissue microarray (TMA) blocks. In addition, 6 cases of oropharyngeal SqCC with previously confirmed hrHPV infection (using both p16 IHC and HPV DNA-ISH) were included in the TMA construction as positive controls. Three punch cores of 1.0 mm were used to transfer per case (ie, from donor blocks) to the TMA (ie, recipient blocks), yielding a total of 4 TMA blocks.

2.3. IHC for $p16^{INK4a}$

Each TMA block was sectioned at 5 μm to assess for the expression of $p16^{INK4a}$ by IHC. The resulting FFPE unstained slides were deparaffinized through standard settings. Pretreatment consisted of CC1 Mild Cell conditioning (EDTA for 30 minutes) with Ventana Ultraview detection system (Ventana Medical Systems, Tucson, AZ) run on Ventana XT Benchmark autostainer. The Ventana predilute CINtec p16 Histology (mouse monoclonal antibody clone E6H4; IVD; catalog no. 725-4713) was used as the primary antibody (32 minutes at 37°C).

The expression of p16 was considered positive when both nuclear and cytoplasmic staining were present in 70% of neoplastic cells or more [18].

2.4. DNA in situ hybridization

Each TMA block was sectioned at 5 μm to assess for the presence of hrHPV DNA. The FFPE sections underwent depraffinization (EZ prep on Ventana XT), cell conditioning (Ventana Cell Consitioner 2) with CC2 (citrate) pretreatment (28 minutes at 95°C), ISH Protease 3 digestion (4 minutes at 37°C), denaturation (12 minutes at 95°C), hybridization