A Comparison of p16INK4A Immunohistochemistry, Chromogenic in situ Hybridization, and Polymerase Chain Reaction Genotyping for Screening for Human Papillomavirus in Basaloid Squamous Carcinoma

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Introduction

Human papillomaviruses (HPV) can promote tumorigenesis at various anatomical sites in the body. Screening for dysplastic changes caused by HPV has decreased the morbidity and mortality due to invasive cervical cancer. Over the last two decades, HPV detection has included molecular methods as an adjunct to traditional Papanicolaou test for cervical cancer screening. HPV-positive HNSSC, specifically high risk HPV-16, have been recognized as a distinct molecular, behavioral and clinical subtype than HPV-negative tumors and therefore the importance of HPV detection in the head and neck region is becoming clear. On the molecular level, oncogenic HPV has two viral genes that synergistically cause deregulation of the cell cycle and malignant transformation. Specifically, viral proteins E6 and E7 act to degrade p53 and retinoblastoma gene family proteins (Rb), respectively. Inhibition of these tumor suppressor genes provokes a cascade of events that causes two points of interest: cell proliferation that escapes normal DNA check points and p16INK4A over-expression. Physiologically, p16INK4A is a cyclin-dependent kinase inhibitor that acts to offset the downstream actions of E6. With increase E6 activity, there is increase p16INK4A expression. Through another mechanism, with the degradation of Rb by E7, the transcription factor E2F is released and this also promotes p16INK4A synthesis. The purpose of this study was to evaluate correlation of HPV detection when using polymerase chain reaction (PCR), chromogenic in situ hybridization (CISH) or p16 immunohistochemistry (IHC), to determine p16INK4A could be used as a surrogate marker for HPV detection.

Methods

Forty BSCC formalin-fixed, paraffin-embedded (FFPE) tissue blocks (18 from head and neck sites, seven from lung, and 15 from anogenital sites) were recovered from Fletcher Allen Health Care pathology archives.

HPV Genotyping

PCR was performed for a β-globin fragment ~200 base pairs (bp) is size to confirm DNA extract suitability for PCR amplification. DNA samples (~100ng) were assayed for HPV using GP5+/6+ primers and a touchdown PCR approach as previously described. Following PCR, an aliquot of the product was screened by agarose gel electrophoresis for the presence of a ~150 base pair (bp) amplicon. Detection of this fragment was taken as evidence of an HPV positive test. HPV genotyping of the 150 bp fragments was performed by dot blot hybridization.

p16INK4A immunohistochemistry (IHC)

Tissues were stained with mouse IgG anti-p16 (clone J8C, Lab Vision, Fremont, CA). Immunohistochemistry (IHC) was performed as previously described, and according to the supplier’s instructions. Negative controls were performed substituting the p16INK4A antibody with a mouse IgG antibody directed against a non-mammalian protein. p16INK4A antibody staining was classed in terms of pattern and intensity. Staining pattern indicated the percentage of tumor cells that were stained and intensity indicated the strength of the stain as negative, weak, or strong (Figure 1).

Chromogenic in situ hybridization (CISH)

CISH utilizing tyramide amplification was performed as previously described on tissue from the same 40 blocks. CISH signals were reviewed and classified as ‘diffuse’ when nuclei were completely stained, (indicative of epimodal HPV), as ‘punctate’ when distinct strong dots were noted within nuclei, (indicative of integrated HPV), as mixed ‘diffuse and punctate’, or as negative. Negative controls tests were performed by omitting the HPV probe from the hybridization mix (Figure 2).

Results

Of 25 head & neck/lung BSCC, 18 (72%) tested HPV-16 positive by PCR and/or HPV positive by CISH; 12 (48%) were CISH positive. p16INK4A staining was identified in 13 (52%) of the 25 head & neck/lung samples, 12 of which were CISH positive. Of 15 anogenital BSCC, 14 (93.3%) were HPV positive by PCR and CISH; 13 for HPV16 and 1 for HPV-33. All 14 (100%) HPV positive anogenital tumors were strongly positive for p16INK4A staining, whereas the HPV negative sample was weakly positive for p16INK4A staining.

Using all three methods (PCR, CISH, IHC) across all samples, there was agreement on HPV status in 33/40 tumors. Of the remaining seven cases, six were positive by PCR but negative by both CISH and p16 IHC and one was negative by PCR but positive by both CISH and p16 IHC. ANOVA was conducted between the three test result groups and yielded no significant differences (F = 0.218, P = 0.354).

Excellent correlation was observed between CISH and p16 IHC (r = 1.00, t-test for significance of the coefficient = 76760.89), and there did appear to be direct correlation between results of PCR and CISH, and PCR and p16 IHC (r = 0.61 for each comparison, t-test for significance of the coefficient = 4.73).

Discussion

Within the volumes of data published regarding oncogenic HPV infection, there is a wide range of incidence reported, ranging from 0 – 100%. Two factors contributing to this spectrum of HPV involvement are the site of the cancer, and the method used for detection. PCR is a frequently employed method, being widely available, well-described, and extremely sensitive; however, it is this sensitivity that may actually limit its clinical utility in certain situations. Simple detection of HPV DNA within a tumor or other cell does not always indicate clinically relevant infection, that is, the virus may not be biologically active in the tumor. This is further supported by Braakhuis, et al., who identified transcriptionally active HPV as a distinct subgroup. This extreme sensitivity of PCR is a potential explanation for the six cases that were PCR positive but both p16 IHC and CISH negative in this study.

This study focused on the use of p16 IHC, PCR and CISH in the BSCC subset, whereas much of the prior literature focused on traditional SCC. Such a comparison study of BSCC demonstrates similar efficacy of p16 IHC, CISH and PCR as observed in SCC, but also demonstrates the etiologic role of HPV in anogenital BSCC.

Within our sample of BSCC there was excellent agreement (100%) between p16 IHC and CISH. In examining correlation of p16 and CISH in anal intraepithelial neoplasia (AIN), Bernard, et al., documented substantial agreement between the two methods, however earlier work on AIN by Kong, et al., showed p16 to be superior in detecting clinically significant HPV infections in AIN. While similar comparison studies in head and neck tumors have not, to our knowledge, been conducted, extrapolating data from the AIN literature lends credence to utilizing p16 IHC as the initial screening test for HPV association regardless of the anatomical site involved by tumor. It appears to be at least equally effective as more technically complicated methods such as CISH, and is widely available and familiar to many laboratories.

References