Experimental and Molecular Pathology 99 (2015) 335–340

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Human papillomavirus E7 serology and association with p16 immunohistochemistry in squamous cell carcinoma of the head and neck

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ARTICLE INFO

Article history:
Received 19 June 2015
Accepted 22 June 2015
Available online 24 June 2015

Keywords:
Human papillomavirus
Head and neck squamous cell carcinoma
Oropharyngeal carcinoma
Serum HPV E7 antibodies
p16 immunohistochemistry

ABSTRACT

Background: Human papillomavirus (HPV)-positive oropharyngeal cancer is associated with improved survival and treatment response as compared to HPV-negative cancers. P16 overexpression is widely accepted as a surrogate marker for HPV positivity.
Methods: A total of 92 serum samples from 75 head and neck squamous cell carcinoma (HNSCC) patients were examined for HPV16 and 18 E7 antibodies by ELISA. Available tissue was tested for HPV–DNA by PCR, and p16 immunohistochemistry was obtained from a deidentified database.
Results: Of 75 HNSCC patients, 25 were HPV E7 seropositive. Seropositivity was strongly associated with cancers of the oropharynx, and correlated with positive p16 immunohistochemistry (IHC) and HPV–DNA. Post-treatment serum was available in a limited subset of patients, revealing a decrease in antibody titers following response to treatment.
Conclusions: HPV E7 seropositivity correlated with positive tumor HPV–DNA and p16 expression, and was strongly associated with cancers of the oropharynx. E7 serology warrants further study as a potential biomarker in HPV-positive HNSCC.

1. Introduction

Head and neck squamous cell carcinomas (HNSCCs) arise in the mucosa of the upper aero-digestive tract including oral cavity, nasopharynx, oropharynx, hypopharynx and larynx. They represent about 3% of all cancers in the United States and are the sixth most common malignancy and the eighth leading cause of cancer-related deaths worldwide (Boscolo-Rizzo et al., 2013; Masood et al., 2013; Parkin et al., 2005). A subset of HNSCC, oropharyngeal cancer, has been found to be strongly associated with human papillomavirus (HPV) infection, particularly high-risk HPV subtypes 16 and 18. Other risk factors for HNSCC are tobacco and alcohol use. Although the prevalence of smoking is decreasing in the United States (CDC data), there has been a recent increase in the incidence of HPV-positive oropharyngeal cancers (Chaturvedi et al., 2011). About 72% of oropharyngeal cancers are caused by HPV in the US (Chaturvedi et al., 2011). It is estimated that around 2000 and 9000 new cases of HPV-associated oropharyngeal cancers are diagnosed in women and men, respectively, each year in the United States, which now outnumber the cases of oropharyngeal cancers attributable solely to smoking or alcohol use (CDC last updated June 5, 2014).

HPV-related HNSCC are distinct entities in etiology and treatment response compared to HPV-negative HNSCC. Patients with HPV-positive oropharyngeal cancer display a better response to treatment and improved prognosis. These tumors are molecularly distinct from HPV-negative HNSCC (Chen et al., 2012; Gillison, 2004). The p16 protein is overexpressed in HPV-positive tumors when HPV oncoprotein E7 inactivates the tumor suppressor RB protein (Gillison, 2004). P16 overexpression, as detected by IHC, is frequently used as a surrogate marker of HPV infection. HPV DNA detection (by *in situ* hybridization or PCR) and p16 IHC are currently used to determine HPV status. Both of these tests require cancer tissue samples. Novel diagnostic techniques using serum samples could carry the potential of earlier and non-invasive methods of detection.

Studies have been done to evaluate serologic markers of HPV infection in head and neck cancer. HPV serology studies have focused on

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http://dx.doi.org/10.1016/j.yexmp.2015.06.018
0014-4800/

detecting HPV16 antibodies and have focused mostly on the L1 protein (Coseo et al., 2011; Ferguson et al., 2006; Herrero et al., 2003; Kirnbauer et al., 1994; Schwartz et al., 1998; Smith et al., 2007), with few studies involving E6 and E7 oncoproteins (Anderson et al., 2011; Furniss et al., 2007; Herrero et al., 2003; Schwartz et al., 1998; Smith et al., 2007) or E1/E2 proteins (Anderson et al., 2011). Antibodies to HPV16 E6 and/or E7 are suggested to be more specific for HNSCC than L1 antibodies (Herrero et al., 2003; Smith et al., 2007). Presence of HPV L1 and E2 antibodies may indicate benign infection whereas E6 and E7 antibodies are more specific for premalignant and malignant lesions. Antibodies against E6 and E7 proteins are largely HPV type-specific. HPV18 has been also detected in HNSCC but few HPV serology studies have examined the seroprevalence of HPV18 in HNSCC (Furniss et al., 2009; Zumbach et al., 2000). Some studies have suggested a correlation between HPV16 E6/E7 seropositivity and clinical outcome of HNSCC patients (Koslabova et al., 2013; Liang et al., 2012; Lopez et al., 2014; Rubenstein et al., 2011; Smith et al., 2008).

Our study examined the presence of HPV16 and 18 specific antibodies to E7 oncoprotein in sera of HNSCC patients and compared the seropositive status with cancer anatomic site, p16 IHC positivity, HPV tumor DNA, smoking status and treatment outcome. Currently, there are no standard commercially available serologic tests that detect HPV-antibodies against any HPV proteins. Although HPV serological studies have been reported; most of them have focused on HPV16 antibodies (not HPV18) and fewer have tested serology both before and after treatment of HNSCC patients.

2. Material and methods

2.1. Serum and tissue samples

The study was approved by the Institutional Review Board at the University of Louisville. Patients who attended the Head and Neck multidisciplinary clinic for evaluation of their cancer were asked to provide written consent. Blood was collected from pathologically confirmed HNSCC patients during the period from 08/27/2009 to 07/30/2014. Blood was let to stand at room temperature for 30 min to clot, and then was centrifuged at 3000 rpm for 10 min to separate serum. Serum aliquots, which were deposited into the Cancer Database and Specimen Repository (CDSR) at the James Graham Brown Cancer Center, were provided to our laboratory at 4 °C. We analyzed 92 de-identified serum samples (including 17 follow-up sera) from 75 patients. HNSCC was classified into one of five categories according to anatomical subsites: a. Oral cavity (includes lip, anterior tongue, gum, floor of mouth, hard palate); b. Oropharynx (includes base of tongue, lingual tonsil, soft palate, uvula, tonsil, and oropharynx); c. Hypopharynx (includes pyriform sinus, hypopharynx); d. Larynx (includes glottis, supraglottis, subglottis); and e. Other unspecified cases (includes sites which were not within one of the categories listed above and those of unknown primary cancer). This categorization is based on The International Classification of Diseases (Bramer, 1988), and the method used by Hashibe et al. (2007). The cancer's clinical stage was classified according to the TNM classification system, 7th edition (Paleri et al., 2010). Demographic and disease characteristics of patients were collected from the CDSR and the researchers were blinded about the HPV status of the patients before serological analysis. Sera from two cervical cancer patients who were seropositive for HPV-16 or 18 were used as positive controls. Formalin-fixed paraffin-embedded (FFPE) tumor samples were available for 11 HNSCC cases for HPV–DNA detection.

2.2. Antigen

Previously, E7 oncoproteins of HPV16 and 18, fused with MBP (maltose binding protein), were expressed in *Escherichia coli* and purified on amylose column (Storey et al., 2013). These fusion proteins were employed as antigens for ELISA in this study.

2.3. Serological analysis by ELISA

Total IgG antibody against E7 oncoproteins of HPV16 and 18 were examined by direct ELISA using recombinant E7 proteins as antigen. Briefly, 500 ng protein/ well in 100 μl of 50 mM bicarbonate buffer (pH 9.6) was coated onto Immulon™ ELISA microplates (Thermo Scientific, USA) for overnight at 4 °C. After three washings with 200 μl of PBS, wells were blocked with 100 μl of 5% PBS-A (PBS containing 5% bovine serum albumin) and incubated for 1 h at 37 °C. Wells were then washed three times with 200 μl of PBS. Sera diluted to 1/100 in 1% PBS-A was added for 1 h at 37 °C followed by the alkaline–phosphatase-(AP-) conjugated goat anti-IgG of human at 1/1000 dilution in 1% PBS-A for 1 h at 37 °C. After the addition of 100 μl of AP-chromogenic substrate (Sigma-104 p-nitrophenyl phosphate substrate; Sigma, St Louis, MO), the absorbance was measured at 405 nm.

All the serum samples were analyzed at the same time to avoid experimental variations. ELISA for all samples was performed three times. Positive control human sera, which had been previously tested as positive for HPV16 or 18 E7 oncoproteins, were employed for each experiment. Wells without antigen or serum was used as negative control and wells containing only substrate were used as background control.

2.4. HPV–DNA detection in HNSCC tissues

We analyzed 11 HNSCC tissues for HPV–DNA detection and typing. FFPE tumor samples were deparafinized using xylene and washed using ethanol, as previously described (Desai et al., 2009). DNA was extracted using DNAeasy Blood & Tissue kit, Qiagen, USA as per the manufacturers' instructions. HPV–DNA detection was performed by PCR using common primers specific for well-conserved L1 region of HPV (My09/11 and GP5 +/GP6 + primers). Both primer sets are capable of binding DNAs of many mucosotrophic HPVs, including HPVs 16 and 18. To measure quality of extracted DNA, β-globin was separately amplified using a specific set of primers. The reaction conditions were as follows: 50–100 ng of template DNA, HiFi PCR buffer, 50 mM $MgSO_4$, 10 mM dNTPs, 20 μM of each primer and 1 U of HiFi Taq polymerase (Invitrogen) in total volume of 20 μl reaction mixture. PCR reaction consisted of an initial incubation of 2 min at 94 °C, followed by 44 cycles of 94 °C (45 s), annealing step at 56 °C (for MY product) or 48 °C (for GP product) (45 s), and 68 °C (45 s) and a final elongation step of 1 min at 68 °C in a MG-96 MyGene™ Peltier Thermal Cycler. The PCR products were analyzed by electrophoresis on a 3% agarose gel stained with ethidium bromide and observed under ultraviolet light. PCR products on gels were purified using QIAquick Gel Extraction Kit (Qiagen, USA) and sent for DNA sequencing to the DNA Core Facility at University of Louisville. Sequences obtained were analyzed using NCBI BLASTn server and HPV type was identified. HPV–DNA status of the tumor tissue was correlated with HPV E7 seropositivity.

2.5. P16 immunohistochemistry

P16 immunostains were performed as per standard of care by the pathologist. P16 IHC was considered positive if there was strong and diffuse staining present in >70% of the malignant cells. The results of the p16 IHC were provided by the CDSR. There were cases in which p16 IHC was not performed as part of a patient's standard diagnostic workup and hence were unavailable at the time of our analysis.

2.6. Statistical analysis

The seropositive and seronegative groups were determined by two different methods regarding the distribution of absorbance values. In the first method, seropositivity was determined as the samples whose absorbance values were 2 standard deviations (SD) from the mean value. We noticed that there was no difference in demographics for