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Antibodies against human papillomavirus proteins in Barrett's dysplasia and intramucosal esophageal adenocarcinoma

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High-risk human papillomavirus (HPV) types 16/18 have been associated with Barrett's dysplasia (BD)/esophageal adenocarcinoma (EAC). Nevertheless, no data exist in relation to serological analysis for HPV antibodies in BD/EAC with site-specific viral DNA status. We prospectively examined antibodies to multiple HPV types in 438 patients representing hospital/reflux controls and Barrett's metaplasia (BM)/BD/intramucosal EAC. Antibody responses to HPV6/11/16/18/31/33/45/52/58 were analyzed using multiplex serology, including antibodies to E6/E7/E1/E2 and L1 antigens. Seropositivity for individual HPV proteins was infrequent in both cases and controls and was ≤10.2%. There was no difference in the seroprevalence of antibodies to any HPV antigen/antibody combination between reclassified cases (BD/EAC) and controls (hospital/reflux/BM) or between HPV16 or HPV18 DNA cases and controls, respectively. Among HPV16 DNA–positive BD/EAC cases, antibodies to HPV16 E7 were significantly more prevalent (3/26, 11.5%) than in hospital and reflux controls plus BM (5/328, 1.5%) (adjusted OR = 10.12, 95% CI: 1.61–63.73, $P = 0.014$). Among HPV18 DNA–positive cases, antibodies to HPV18 E1 were present in 3/6 (50%) cases versus 5/328 (1.5%) controls (adjusted OR = 44.28, 95% CI: 6.10–321.47, $P = 0.0002$). Although antibodies against HPV were generally uncommon in cases and controls, immune responses against two early proteins of HPV16/18 were significantly more frequent in viral DNA–positive BD/intramucosal EAC.

Keywords: Barrett's esophagus; esophageal cancer; human papillomavirus; serology; immune response; gastroesophageal reflux disease

Introduction

Esophageal adenocarcinoma (EAC) is one of the fastest-growing cancers in the Western world,[1,2] though recently, the rate of increase has diminished and possibly plateaued in the United States and Sweden.[1,3,4] In this regard, the recent discovery of a strong association of transcriptionally active high-risk human papillomavirus (hr-HPV) with Barrett's dysplasia (BD) and EAC (but not Barrett's metaplasia (BM)) should be further investigated.[5]

In a cross-sectional study, it has been demonstrated that hr-HPV viral load and integration status is greater in BD and EAC as compared with BM.[6] Moreover, persistent hr-HPV infection and p53 overexpression have been associated with treatment failure after endoscopic ablation of BD and EAC.[7] Interestingly, HPV-associated EAC has a

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distinct distribution of molecular aberrations/genomic abnormalities compared with HPV-negative EAC, indicating different biological mechanisms of tumor formation.[8] Additionally, we have recently shown that aberrations of the retinoblastoma protein pathway, that is, upregulation of $p16^{INK4A}$ and downregulation of pRb, as well as wild-type p53 are hallmarks of active HPV involvement in BD and EAC.[9] Intriguingly, HPV-positive high-grade BD and EAC seem to be distinct biological entities with a favorable prognosis compared with viral-negative esophageal tumors and may benefit from treatment de-escalation.[10] This is analogous to HPV-induced head and neck squamous cell carcinomas (HNSCCs) being a distinct subset with improved survival compared with viral-negative oropharyngeal malignancies.[11,12]

Nevertheless, prior negative studies using polymerase chain reaction (PCR) techniques exist owing to poor tissue classification, suboptimal testing methodology (including primer selection) compounded by low viral load, and racial and geographic variations.[13–15] The study by Antonsson *et al.* found no evidence of HPV in 233 EAC tissue specimens.[16] This study's shortcomings included the use of formalin-fixed tissue to detect HPV, which is an inferior method compared with fresh frozen tissue (FFT).[16,17] Moreover, some tissue specimens used in the study were more than 10 years old, which can result in DNA invalidity. In particular, the MY09/MY11 may not amplify the 450-bp target area. Tissue fixation with formalin and length of storage adversely affects DNA quality and much of this damage is irreversible. We have previously demonstrated that formalin-fixed paraffin-embedded tissue (FFPET) will yield a false-negative PCR result for HPV DNA in 60% of cases and as such we strongly recommend the use of FFT.[7] Nevertheless, some argue that DNA and RNA can be reliably detected even in FFPET blocks if appropriately handled using highly sensitive methods. Another important reason as to why it is difficult to detect HPV DNA in BD/EAC is the low viral load in glandular tissue; very much less than in the uterine cervix, anal canal, and oropharynx, which consist of squamous epithelium.[6]

Some of these misclassified as "negative" studies used nondysplastic BE either exclusively or in combination with adenocarcinoma (AC) and control tissue.[13–15] Metaplastic tissue is not associated with HPV.[5,13,14] In this regard, a case–control study by El-Serag *et al.* found no viral presence in 127 patients with BE in the North American patient population.[13] Rai *et al.* again confirmed the virtual absence of HPV infection in BM in the UK population.[14] In another study, Iyer *et al.* found no difference in the HPV prevalence between BE (27.4%), EAC (31%), and controls (20.7%), and thus concluded that it was of no etiologic significance.[15] Cross-contamination may well have been the cause of this anomaly.

Conversely, a systematic review has reported HPV prevalence rates of 35% in EAC ($n = 174$), not dissimilar to our findings.[18] Another systematic review, which included 19 studies, concluded that the pooled prevalence of HPV in EAC was 13%. The authors suggested that the low prevalence rate may have been caused by small sample sizes and compromised detection methods.[19]

There is a differential gene expression during the varied phases of the HPV life cycle in the run-up to malignancy, which may generate a host immune response. High antibody titers to the HPV16 L1 have been associated with the archetypal viral-driven cancers of the cervix[20,21] and oropharynx.[22,23] In a nested case–control study involving 292 head and neck cancers and 1568 controls, Mork *et al.* reported that HPV16-seropositive subjects (antibodies against L1 and L2) had 14 times the risk of oropharyngeal malignancy and three times the risk of tongue cancer as compared with HPV16-seronegative individuals.[24] Similarly, seropositivity for HPV16 E6 and E7 antibodies was associated with 73 times the risk of oropharyngeal tumors as compared with those testing negative for these antibodies.[25] Not surprisingly, antibodies against E6 and E7 of HPV16 and 18 are highly specific for cervical malignancy when compared with control subjects.[26–28]

Interestingly, a significant association with increased IgG antibodies to HPV16 has been reported in esophageal cancer (EC; unclassified histology) (OR for medium antibody level = 1.3, and OR for high antibody level = 1.6, $P = 0.002$).[21] In the case of squamous cell carcinoma of the esophagus (ESCC), the association with HPV is yet to be proven.[29]

There is no information regarding the immune response to HPV E6 and E7 proteins of genotypes 16 and 18 or other high-risk types and to other early

or late viral proteins in Barrett's esophagus (BE), both metaplastic and dysplastic, and EAC. Thus, this robust investigation involving multiplex HPV serology was undertaken.

We were particularly interested in the early proteins, that is, E6 and E7 (induction and maintenance of cellular transformation), and the late viral capsid protein, namely, L1 (the marker of cumulative exposure to the virus) in patients with reflux disease BE (metaplastic and dysplastic) and early EAC. We hypothesized that HPV antibodies would be detected only late in the BD–BD–AC sequence given the low viral load in esophageal tissues.[6,10] Intriguingly, HPV antibodies are detected late in cervical intraepithelial neoplasia (CIN), carcinoma *in situ* (CIS), and invasive cancer, even though the viral load is much higher in the cervix.[28,30,31] By contrast, antibodies to HPV16 appear approximately 10 years prior to the diagnosis of oropharyngeal cancers (OPCs).[24] We, therefore, studied HPV E6/E7 antibodies as well as those against E1 plus E2 regulatory proteins (uncertain significance), and the L1 antigen of the virus in plasma samples of patients with BM/BD and intramucosal AC; and we compared them with gastroesophageal reflux disease (GERD) and hospital controls. Additionally, the analysis was performed by defining cases as BD/EAC and controls as hospital, GERD, and BM subjects, given a proven lack of viral involvement with nondysplastic BE.[5,6,9] Correlation of these antibodies with the lesional tissue HPV DNA was also performed.

Materials and methods

Study design

A cross-sectional method was used to estimate the prevalence of antibodies against the HPV proteins E6, E7, E1, E2, and L1 in human plasma in hospital patients detailed below. In addition, viral DNA was analyzed from fresh frozen biopsy samples. HPV transcriptional activity was assessed via $p16^{INK4A}$ immunohistochemistry (IHC) and E6/E7 mRNA *in situ* hybridization (ISH).

The patients were prospectively recruited and were representative of hospital and GERD controls, as well as the BM–BD–AC sequence. All subjects underwent gastroscopy either in a regional hospital (Launceston General Hospital, Launceston, Tasmania) or a tertiary referral center (Bankstown-Lidcombe Hospital, Sydney, Australia) between July 2008 and July 2015. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained from the Human Research Ethics Committee, Tasmania (H9304) and South Western Sydney Local Health Network (HREC/11/LPOOL/42, LNR/12/LPOOL/221, and LNR/12/LPOOL/131).

Patient selection and specimen collection

Patient selection. Patients satisfying the inclusion and exclusion criteria documented below were recruited into this study. They had been referred from the specialist clinics or their general practitioners to the open-access endoscopy units of either hospital.

Following written consent prior to endoscopy, age, gender, weight, height, smoking (ever or never) and alcohol histories (ever or never, and excess >21 units per week for men, and >14 units per week for women), and medications (nonsteroidal anti-inflammatory drugs (NSAIDs)/aspirin and proton pump inhibitors (PPIs)) were recorded.

Inclusion criteria. These included all patients aged 18–90 years undergoing gastroscopy for investigation of suspected GERD and/or its complications, Barrett's surveillance, and those patients reporting for endoscopic ablative therapy. Hospital controls were those undergoing gastroscopy for anemia/occult blood–positive stools, chronic diarrhea requiring small bowel biopsy, unexplained nausea/vomiting, atypical chest pain, and lower abdominal pain/irritable bowel syndrome. Exclusion criteria included emergency gastroscopy, prior upper gastrointestinal malignancy/surgery, history of peptic ulcer disease, post-HPV vaccination or with a current or previous history of an HPV-associated lesion, immunosuppressed patients, known human immunodeficiency virus (HIV) positive, inability to give informed consent, or any contraindication to performing endoscopic biopsies. Potential hospital control patients were excluded if they had dyspepsia or consumed antacids/antisecretory therapy in the past 2 years.

Disease outcome classification. Six *a priori*–defined patient categories were as follows: (1) hospital controls (no clinical, endoscopic, or histopathological evidence of reflux disease or dyspepsia), (2) GERD controls (erosive and nonerosive disease without BE), and (3) short- (<3 cm columnar

epithelium) and long-segment (≥3 cm columnar epithelium) BE, BD, and EAC cases. Additional statistical analysis was undertaken by defining cases as BD/EAC and controls as hospital, GERD, and BE patients given the lack of viral involvement in nondysplastic BM, as described earlier. Patients with GERD had at least twice weekly attacks of heartburn and/or acid reflux as their dominant complaint during the month before endoscopy. The presence of BE was defined as columnar epithelium with specialized intestinal metaplasia obtained from any length segment of the tubular esophagus. The length of BE and the presence and grade of dysplasia were documented. The pathologists performing the histopathological examination and the virologists carrying out the HPV detection experiments were blinded to the clinical diagnosis and other findings. Slides were examined by two experienced gastrointestinal pathologists at a central reporting laboratory of a major metropolitan teaching hospital. For the purposes of this analysis, the hospital and GERD controls were combined into a single group, and the short- and long-segment groups were also combined.

Specimen collection

In all patients, in addition to clinically indicated tissue specimens, two biopsies each were obtained from within the Barrett's columnar/dysplastic/AC segment, or in the case of GERD and hospital controls, from the corresponding position in the squamous epithelium, that is, 2 cm above the gastroesophageal junction using separate forceps for each of the biopsies. In patients with BE, BD, and endoscopically resected intramucosal EAC, chromoendoscopy-assisted targeted biopsies were obtained. The samples were placed on dry ice and transported to the laboratory for DNA isolation, and the other tissue biopsy was formalin-fixed. In addition, 3 mL of blood was collected from each patient.

Study population

Four hundred and thirty-eight patient sera were analyzed: 225 combined hospital and GERD controls, 103 BE, 30 low-grade dysplasia (LGD), and 80 high-grade dysplasia (HGD)/EAC. Four hundred and seven patients and controls had available FFT for HPV DNA analysis, which was collected at the same time point as the blood samples.

Laboratory studies

DNA isolation, PCR, viral genotyping, E6/E7 mRNA ISH, and $p16^{INK4A}$ IHC. HPV detection in genomic DNA was performed by nested PCR amplification of the viral L1 gene by using MY09/MY11 and $GP5^{+}/6^{+}$ primers.[5] HPV genotypes were determined by sequencing.[5] Great care was undertaken to prevent cross-contamination when sectioning tissue blocks for DNA isolation, RNA ISH, and $p16^{INK4A}$ IHC. The first section was discarded, and each tissue specimen was handled with new gloves and sectioned with a new blade. The sectioning area was cleaned with acetone and 70% alcohol. To guard against systematic contamination of PCR reagents, positive (HPV16 DNA- and RNA-positive cervical cancer (CC)) and negative (deionized water and PCR master mix without template) controls were included in PCR. All positive and negative controls reacted as such in all assay runs. Filter tips were used for all pipetting. Furthermore, separate rooms were used for preparing the PCR reaction mix, template handling, gel electrophoresis, and performing nested reactions. Routine decontamination by UV irradiation was performed in the DNA-free PCR hood before each run. PCR was run thrice to confirm result reproducibility. E6/E7 mRNA was performed manually using the RNAscope® 2.5 high definition assay (Advanced Cell Diagnostics Inc; Hayward, CA) with a cocktail of probes targeting 18 hr-HPV types, as previously described.[9] $P16^{INK4A}$ IHC was performed on FFPET using the CINtec® Histology Kit (Heidelberg, Germany).[9]

Multiplex serological analyses

Three milliliters of whole blood was collected in EDTA tubes and spun down within 12–24 h to obtain serum, which was stored at −80 °C. Sera were shipped on dry ice to the German Cancer Research Center (DKFZ, Heidelberg, Germany) for multiplex serological analysis by laboratory scientists blinded to the diagnosis of the subjects. Testing was performed using a glutathione S-transferase capture immunoassay in combination with fluorescent beads to detect antibodies against the major capsid protein L1, early proteins E1 and E2, and early oncoproteins E6 and E7 for the following HPV types: high-risk (oncogenic) mucosal types HPV16 and HPV18 (L1, E1, E2, E6, and E7) and HPV31, 33, 45, 52, and 58 (L1, E6, and E7); and low-risk

Table 1. Clinical characteristics of study subjects

	Cases (BE/BD/EAC; $n = 213$)	Controls (hospital and GERD; $n = 225$)	P value
Age	62.1 (14.3)	55.2 (16.7)	<0.0001
Gender			<0.0001
Male	171 (81.0%)	92 (42.0%)	
Female	40 (19.0%)	127 (58%)	
Smoking			0.002
Yes	135 (64.3%)	107 (49.3%)	
No	75 (35.7%)	110 (50.7%)	
Alcohol			0.0004
Yes	148 (70.5%)	117 (53.9%)	
No	62 (29.5%)	100 (46.1%)	
Excess alcohol			<0.0001
Yes	38 (18.1%)	9 (4.2%)	
No	172 (81.9%)	208 (95.8%)	
BMI	27.7 (5.2)	27.5 (6.6)	0.77
Hiatal hernia			<0.0001
Yes	111 (56.9%)	35 (16.2)	
No	84 (43.1%)	181 (83.8%)	
Esophagitis			<0.0001
Yes	67 (35.1%)	25 (11.6%)	
No	124 (64.9%)	191 (88.4%)	
PPI			<0.0001
Yes	138 (66.0%)	90 (41.9%)	
No	71 (34.0%)	125 (58.1%)	
NSAIDs			0.26
Yes	44 (21.0%)	36 (16.7%)	
No	165 (79.0%)	179 (83.3%)	
Statins			0.38
Yes	56 (26.9%)	50 (23.3%)	
No	152 (73.1%)	165 (76.7%)	

BE, Barrett's esophagus; BD, Barrett's dysplasia; BMI, body mass index; EAC, esophageal adenocarcinoma; GERD, gastroesophageal reflux disease; PPI, proton pump inhibitor; NSAIDs, nonsteroidal anti-inflammatory drugs.

(nononcogenic) mucosal types HPV6 and 11 (L1, E6, and E7).

Median fluorescence intensity (MFI) values were measured and categorized as antibody positive or negative for all serological markers that were assessed.[30–33] This was based on predefined cutoffs (mean $\pm$ 5 SD for early HPV proteins and mean $\pm$ 3 SD with the exclusion of positive outliers for late HPV proteins) of MFI values obtained from sera of a group of female Korean virgins who were HPV DNA negative.[34] The minimum cutoff for seropositivity was 200 MFI. In the case of HPV6 E6, HPV11 L1, and HPV33 E7, the cutoff was arbitrarily set at 500 MFI to increase specificity.[35] The measurement standard was a reference serum with known reactivity to several antigens (HPV16 L1, E6, and E7). Multiplex HPV serology has been shown to be highly reproducible, yielding both Pearson's and intraclass correlation coefficients of >0.9.[32,36]

Statistical analysis

Differences between cases and controls in regard to baseline characteristics were assessed using a two-sample t-test for all numerical data and chi-square analysis for binary measurements. Seropositivity was reported as a percentage. OR and 95% CIs were estimated for HPV16 and 18 and other high-risk type (after excluding coinfection with HPV16 and/or 18) antigens in (1) all cases, (2) HPV genotype DNA–positive patients (either 16, 18, or other high-risk types assessed independently), and (3) HPV genotype–negative subjects (for 16, 18, or other high-risk types assessed separately), each compared with controls. For hr-HPV other than

Table 2. Seropositivity rate between combined control group (hospital and GERD controls, and BE) and BD/EAC cases

	BD + EAC ($n = 110$)/ CON + BE ($n = 328$)	OR adjusted[a] (95% CI)	*P* value adjusted[a]
Any 16/18 E6/7	5 (4.6%)/10 (3.1%)	1.58 (0.42–6.00)	0.50
Any 16-positive	13 (11.8%)/39 (11.9%)	0.74 (0.31–1.75)	0.49
Any 18-positive	15 (13.8%)/26 (7.9%)	1.38 (0.59–3.21)	0.46
Any 16- or 18-positive	22 (20.0%)/54 (16.5%)	0.91 (0.46–1.82)	0.79
Any 31-positive	7 (6.4%)/18 (5.5%)	1.77 (0.54–5.80)	0.34
Any 33-positive	6 (5.5%)/9 (2.7%)	3.71 (0.85–16.12)	0.08
Any 45-positive	4 (3.6%)/11 (3.4%)	1.41 (0.32–6.33)	0.65
Any 52-positive	7 (6.4%)/10 (3.1%)	1.46 (0.43–4.93)	0.54
Any 58-positive	5 (4.6%)/18 (5.5%)	1.07 (0.43–3.63)	0.92
Any 6-positive	11 (10.0%)/34 (10.4%)	1.50 (0.60–3.75)	0.38
Any 11-positive	5 (4.6%)/18 (5.5%)	2.01 (0.46–8.76)	0.35
Any 6 or 11	13 (11.8%)/42 (12.8%)	1.58 (0.67–3.74)	0.30
Any hr-HPV	38 (34.6%)/87 (26.5%)	1.25 (0.71–2.21)	0.45
16 E6 or E7	4 (3.6%)/7 (2.1%)	2.08 (0.43–10.07)	0.36
16 E6 and E7	1 (0.9%)/0 (0%)	NA	NA
18 E6 or E7	1 (0.9%)/3 (0.9%)	0.79 (0.06–10.79)	0.86
18 E6 and E7	0 (0%)/0 (0%)	NA	NA
Any other hr-HPV (31, 33, 45, 52, and 58), E6 or E7 only	14 (12.7%)/30 (9.2%)	1.38 (0.61–3.15)	0.44

[a]Adjusted for age, gender, BMI, alcohol consumption, smoking, esophagitis, HH, NSAIDs/aspirin, statins, and PPI use.

16 and 18, seropositivity was defined as the detection of antibodies for at least one of the HPV31, 33, 45, 52, or 58. Logistic regression was performed to calculate the adjusted OR for known or potential confounders, that is, age, gender, body mass index (BMI), alcohol consumption (ever and excess), smoking history (ever/never), esophagitis, hiatus hernia (HH), and medication use, that is, NSAIDs/aspirin, statins, and PPIs. All statistical tests were performed using SAS® 9.4 (SAS Institute Inc., Cary, NC) and the level of significance was set at 0.05.

Results

Table 1 summarizes the clinical characteristics of a total of 213 participants with esophageal lesions (metaplasia/dysplasia/AC) and 225 controls (combined hospital and reflux subjects). The mean age of BE/BD/EAC was 62.1 years and for controls, it was 55.2 years ($P < 0.001$). As expected, the proportion of males (81%), smoking, alcohol (ever and excess), HH, esophagitis, and PPI use were significantly higher in cases than controls. Among 438 subjects, 437 were Caucasians and one control patient was African.

Seropositivity for individual HPV proteins was infrequent in both cases and controls and did not exceed 10.2% (data not shown). A total of 125/438 (28.5%) patients were seropositive for at least one of the HPV proteins from seven of the most prevalent hr-HPV types: HPV16/18 (E1, E2, E6, E7, and L1) and HPV31, 33, 45, 52, and 58 (E6 and E7) (Table 2). There was no statistical difference between 52 (24.4%) cases and 73 (32.4%) controls (OR = 0.67, 95% CI: 0.44–1.02, $P = 0.06$).

Previous publications have clearly demonstrated that HPV has no biological role in BM.[5,7,9] Thus, for subsequent analysis, BE was grouped with combined controls and compared with BD (LGD and HGD) and EAC (Table 2).

The prevalence of antibodies against HPV16 E6/E7 was 4/110 (3.6%) among cases (BD/EAC) and 7/328 (2.1%) among controls (hospital, GERD, and BE) (OR = 2.08, 95% CI: 0.43–10.07, $P = 0.71$) (Table 2). HPV16/18 E6/E7 antibodies were present in 5/110 (4.6%) patients with BD/EAC and 10/328 (3.1%) controls (OR = 1.58, 95% CI: 0.42–6.00,

Table 3. Seroprevalence for antibodies against HPV16 proteins L1, E1, E2, E6, and E7 among cases with HPV16 DNA positivity and controls

	Controls +BE ($n = 328$)	HPV16 DNA$^+$ (BD + EAC; $n = 26$)	OR adjusted[a] (95% CI)	P value adjusted[a]
HPV16 L1	20 (6.1%)	1 (3.1%)	0.87 (0.10–7.84)	0.90
HPV16 E6	2 (0.6%)	1 (3.9%)	4.23 (0.21–83.31)	0.34
HPV16 E7	5 (1.5%)	3 (11.5%)	10.12 (1.61–63.73)	0.014
HPV16 E1	12 (3.7%)	2 (7.7%)	0.86 (0.09–8.41)	0.89
HPV16 E2	5 (1.5%)	2 (7.7%)	2.87 (0.39–20.92)	0.30
Any HPV16-positive	39 (11.9%)	4 (15.4%)	0.96 (0.25–3.65)	0.95
HPV16 E6 or E7	7 (2.1%)	3 (11.5%)	6.60 (1.19–36.64)	0.03
HPV16 E6 and E7	0	1 (3.9%)	NA	NA

[a]Adjusted for age, gender, BMI, alcohol consumption, smoking, esophagitis, HH, NSAIDs/aspirin, statins, and PPI use. 14/26 HPV16 DNA–positive cases had evidence for viral transcriptional activity.

$P = 0.50$ (adjusted)). For other hr-HPV types (31, 33, 45, 52, and 58), 14 (12.7%) patients with dysplasia/AC, and 30 (9.2%) controls were seropositive for E6/E7 (OR = 1.38, 95% CI: 0.61–3.15, $P = 0.44$), respectively. There were two sera (BE = 1 and HGD = 1) with HPV16 E6 >1000 MFI, which is the best serological standalone marker to identify HPV-driven lesions. The patient with HGD had detectable HPV16 DNA on PCR and mounted strong antibodies to three other early proteins (HPV16 E1, E2, and E7).

HPV concordance between serology and DNA

HPV16. Among 438 patients, esophageal tissue was available in 407 for testing of HPV DNA status. Of these 407 patients, 70 (17.2%) were HPV DNA–positive, that is, combined hospital and GERD controls = 24, BE = 14, BD (LGD and HGD) = 23, and EAC = 9. HPV16 DNA was detected in 26 BD/EAC cases and 13 controls. Among these 26 BD/EAC patients, nine were positive for E6/E7 mRNA activity and 10 had $p16^{INK4A}$ gene (*CDKN2A*) overexpression. Five out of these 19 patients coexpressed $p16^{INK4A}$ and E6/E7 mRNA. Thus, 14/26 (53.8%) cases had evidence for a biologically active virus in the esophageal lesions ($p16^{INK4A}$- and/or E6/E7-positive). None of the controls had transcriptionally active HPV. Tables 3 and 4 depict the seroprevalence of antibodies against HPV16 proteins L1, E1, E2, E6, and E7 among cases classified by HPV16 DNA status in the lesional esophageal tissue and controls. In the case of HPV16 E7, there was a significantly greater prevalence of this antibody in cases (11.5%) as compared with controls (1.5%) (OR 10.12, 95% CI: 1.61–63.73, $P = 0.014$ (adjusted)). Likewise, for HPV16 E6 or E7, the prevalence between cases (11.5%) and controls (2.1%) was statistically significant even after adjusting for confounders (OR = 6.60, 95% CI: 1.19–36.64, $P = 0.03$). Although HPV16 E1 and E2 seropositivity were higher in BD/EAC (7.7%) versus controls (3.7% and 1.5%, respectively), it did not attain significance level after accounting for known confounders, such as age, gender, BMI, alcohol consumption, smoking history, esophagitis, HH, as well as medication use, that is, NSAIDs/aspirin, statins, and PPIs. In the case of HPV16 DNA–negative cases and controls, there was no difference in the seroprevalence of antibodies to HPV16 (Table 4).

HPV18. HPV18 DNA was found in six BD/EAC patients and 17 controls. Among cases, three had detectable E6/E7 mRNA. $P16^{INK4A}$ gene overexpression was also identified in three individuals. One subject coexpressed $p16^{INK4A}$ and E6/E7 mRNA. In total, 5/6 (83.3%) cases were positive for either E6/E7 mRNA or $p16^{INK4A}$ in esophageal lesional mucosa. None of the controls had detectable viral transcriptional activity. Tables 5 and 6 describe the seroprevalence of antibodies to HPV18 proteins L1, E1, E2, E4, E6, and E7 between cases stratified by HPV18 DNA status and among controls. The absolute numbers are very small giving rise to very wide CIs. The only statistically significant result was that of HPV18 E1 that was present in 3/6 (50%) of cases versus 5/328 (1.5%) of controls (OR = 44.28, 95% CI: 6.10–321.47, $P = 0.0002$) (Table 5). The seroprevalence of antibodies against all other HPV18 proteins, such as

Table 4. Seroprevalence of antibodies against HPV16 proteins L1, E1, E2, E6, and E7 among cases with HPV16 DNA negativity and controls

	Control + BE ($n = 328$)	HPV16 DNA$^-$ (BD + EAC; $n = 84$)	OR adjusted[a] (95% CI)	P value adjusted[a]
HPV16 L1	20 (6.1%)	3 (3.6%)	0.79 (0.17–3.63)	0.76
HPV16 E6	2 (0.6%)	0 (0%)	NA	NA
HPV16 E7	5 (1.5%)	1 (1.2%)	2.01 (0.17–23.16)	0.58
HPV16 E1	12 (3.7%)	4 (4.8%)	0.85 (0.20–3.63)	0.82
HPV16 E2	5 (1.5%)	1 (1.2%)	0.23 (0.02–2.72)	0.24
Any HPV16-positive	39 (11.9%)	9 (10.7%)	0.74 (0.28–1.93)	0.53
HPV16 E6 or E7	7 (2.1%)	1 (1.2%)	1.2 (0.11–11.47)	0.92
HPV16 E6 and E7	0 (0%)	0 (0%)	NA	NA

[a]Adjusted for age, gender, BMI, alcohol consumption, smoking, esophagitis, HH, NSAIDs/aspirin, statins, and PPI use.

L1, E2, E6, and E7, was 0% among cases and was no different from controls (L1 = 3.1%, E2 = 2.4%, E6 = 0.3%, and E7 = 0.6%).

The seroprevalence of HPV18 antibodies did not differ significantly between HPV18 DNA–negative cases and controls (Table 6).

HPV31/33/45/52/58. There was the absence in esophageal tissue DNA of hr-HPV mucosal types 31, 33, 45, 52, and 58 in dysplastic and AC lesions.

Discussion

This is the first study to examine the association between BD and EAC plus controls in relation to HPV serological markers, including E6/E7 seroreactivities. Importantly, we correlated the immune response to these viral proteins with the causal type of HPV in the lesion. Moreover, we investigated the relationship between EAC and precancer and antibodies to HPV types other than HPV16 and HPV18, even though the latter accounts for >90% of the genotypes found in the esophageal tissue of BE, BD, and EAC. Generally, antibodies against HPV were infrequent in our study population. The prevalence of antibodies against any HPV16 antigen was 11.9% in our study, which compares favorably with the 15% seroprevalence of HPV16 reported by Antonsson *et al.* in the Queensland population ($n = 390$).[37] Nevertheless, given the availability of information on the HPV DNA status of all cases, we were able to find a 10-fold statistically significant difference between HPV16 E7 in cases (with HPV16 DNA–positive lesions of which over 50% exhibited transcriptional activity) as compared with controls. E6 or E7 antibodies to this genotype were also elevated more than sixfold in BD/EAC versus controls ($P = 0.03$). It is important to note that HPV16 has been incriminated in >80% of virally associated BD and EAC, and HPV18 in the remainder.[5,7] E6/E7 antibodies are regarded as the markers of current HPV-related lesions.[22,35,38–40] More specifically, antibodies to E6 and E7 have been shown to denote an HPV-driven process in invasive cervical malignancy, HNSCC, and penile cancers.[22,27,39,41]

Seropositivity to E1 and E2 antigens of HPV16 was higher in HPV16 DNA–positive cases when compared with controls, but failed to reach statistical significance after adjustment for confounders. This could be due to the small sample size of HPV DNA–positive cases as well as the very early stage of disease or a true failure to seroconvert. In regard to HPV18 DNA–positive subjects, antibodies to E1 were 44 times greater in cases (of whom >80% had the biologically active virus) versus controls ($P = 0.0002$) (Table 5). It is thought that together the early proteins E1 and E2 form a complex necessary for viral replication.

Immune responses to early proteins, but not L1, seem to correlate with esophageal lesions in which DNA of the same HPV type can be detected. L1 is the major component of the HPV capsid and is reflective of cumulative exposure to HPV infection.[42,43] It is not indicative of an HPV-induced malignancy.

In invasive CC, early proteins, especially HPV16 E1 and to a lesser degree E2 (HPV18 E1 and E2 were not analyzed), showed a particularly strong association with CC (OR = 60.9, 95% CI: 8.2–453, and OR = 12.8, 95% CI: 4.8–33.6).[44] Although HPV16 L1 and HPV18 L1 were both positively associated with the CC risk, the ORs between cases and controls were much lower than for E1 and E2 proteins.[44]

Table 5. Seroprevalence of antibodies against HPV18 proteins L1, E1, E2, E6, and E7 among cases with HPV18 DNA positivity and controls

	Control + BE ($n = 328$)	HPV18 DNA$^+$ (BD + EAC; $n = 6$)	OR adjusted[a] (95% CI)	P value adjusted[a]
HPV18 L1	10 (3.1%)	0 (0%)	NA	NA
HPV18 E6	1 (0.3%)	0 (0%)	NA	NA
HPV18 E7	2 (0.6%)	0 (0%)	NA	NA
HPV18 E1	5 (1.5%)	3 (50%)	44.28 (6.10–321.47)[a]	0.0002
HPV18 E2	8 (2.4%)	0 (0%)	NA	NA
Any HPV18-positive	26 (7.9%)	3 (50.0%)	10.10 (1.75–58.09)[a]	0.01
HPV18 E6 or E7	3 (0.9%)	0 (0%)	NA	NA
HPV18 E6 and E7	0 (05)	0 (0%)	NA	NA

[a]Adjusted for gender and BMI. 5/6 HPV DNA–positive cases had evidence for viral transcriptional activity.

Moreover, when compared with seropositivity to early proteins, L1 antibody associations with CC were not much greater in tumors positive for HPV DNA than those that were negative for the same viral genotype. In cervical malignancy, HPV E6/E7 antibodies are late tumor markers that increase progressively with cancer stage.[27,39,45] In a prospective investigation of cervical malignancy, <10% of patients had antibodies to E6/E7 proteins of HPV16/18, compared with 1% of controls.[38] A more recent publication from the EPIC cohort involving 425 CIN3/CIS and 1218 controls followed-up for a median of 9 years revealed no statistical difference for antibodies against E6/E7 for any of the similarly tested hr-HPV genotypes. The only exception was that these cervical lesions were more likely than controls to be seropositive for L1 serological markers of HPV16, 18, and 45. The limitation of this study was that lesions were not stratified by HPV DNA type.[46] Paradoxically, E6/E7 serological markers appear early in HNSCC. It is thought that tonsils are lymphoid tissue rich in antigen-presenting cells would be an easy and early portal for viral processing and generation of an immune response. In fact, HPV E6 seropositivity has been demonstrated to be present >10 years before the diagnosis of head and neck cancers.[35]

In the InterScope study, there were significant associations between ESCC and antibodies to E6 for HPV16 (OR = 1.89, 95% CI: 1.09–3.29, $P = 0.023$) and HPV6 (OR = 2.53, 95% CI: 1.51–4.25, $P < 0.001$), but no other HPV types.[33] The authors concluded that there was limited serological evidence for an association between ESCC and HPV. A follow-up study by the same authors demonstrated a lack of association between HPV and ESCC despite the serological associations.[47] Lagergren *et al.* tested IgG against HPV16/18 capsids in 173 EAC (distal esophageal and junctional), 121 ESCC, and 302 controls, and their only significant (unexpected) finding was a negative association between HPV18 and EAC. The authors attributed it to a possible bias.[48] Furthermore, the esophageal lesions were not tested for HPV DNA, and thus site-specific correlation with serology was not possible. Intriguingly, their results differed substantially from three prior published positive studies, two of which used an identical serological assay on unclassified ECs and controls, though there may have been some study design differences.[49–51] In the latter two studies, HPV16 seropositivity was associated with a six-fold excess risk of esophageal malignancy (mainly ESCC) though again, the HPV DNA status of the esophageal lesion was unknown.[50,51]

High-throughput multiplex serological analysis enabled simultaneous analysis of antibodies against a broad range of hr-HPV. We included the oncoproteins E6 and E7 of multiple hr-HPV types as antibodies against these are considered the markers of HPV-driven cancer.[25–28,35] L1 antibodies denote exposure or cumulative infection markers.[42,43] In addition, we only included E1 and E2 for HPV16 and 18. In the case of HPV16, these antibodies have been shown to be additional cancer biomarkers in OPC.[52] We analyzed E1 and E2 of HPV18 for the sake of completeness. E1 and E2 were only tested for HPV16 and 18 because they are the most frequent genotypes in CC and OPC. Only recently, the E1 and E2 proteins of the rare hr-HPV types have been available. Unfortunately, at the time of the study, they were unavailable for testing.

Table 6. Seroprevalence of antibodies against HPV18 proteins L1, E1, E2, E6, and E7 among cases with HPV18 DNA negativity and controls

	Control + BE ($n = 328$)	HPV18 DNA$^-$ (BD + EAC; $n = 104$)	OR adjusted[a] (95% CI)	P value adjusted[a]
HPV18 L1	10 (3.1%)	4 (3.9%)	1.88 (0.38–9.28)	0.44
HPV18 E6	1 (0.3%)	0 (0%)	NA	NA
HPV18 E7	2 (0.6%)	1 (1.0%)	2.27 (0.11–46.13)	0.59
HPV18 E1	5 (1.5%)	3 (2.9%)	0.86 (0.13–5.69)	0.87
HPV18 E2	8 (2.4%)	3 (2.9%)	0.82 (0.17–4.01)	0.80
Any HPV18-positive	26 (7.9%)	11 (10.6%)	1.08 (0.43–2.73)	0.87
HPV18 E6 or E7	3 (0.9%)	1 (1.0%)	0.83 (0.06–11.33)	0.89
HPV18 E6 and E7	0 (0%)	0 (0%)	NA	NA

[a]Adjusted for age, gender, BMI, alcohol consumption, smoking, esophagitis, HH, NSAIDs/aspirin, statins, and PPI use.

It is difficult to differentiate a true failure of seroconversion from the lack of sensitivity of the assay. Analysis of repeat samples will help determine if the serological response is sustained. Highly sensitive methods were used for HPV DNA detection via nested PCR in FFT and viral transcriptional activity in FFPET. The availability of HPV DNA genotype data on almost all cases made it possible to demonstrate that antibody responses to HPV16 E6/E7 and other early proteins were specific to the esophageal lesions containing the same viral genotype. The prospective nature of this investigation resulted in a comprehensive clinical data set on almost all the subjects enrolled in this study. This allowed for adjustments of general and study-specific confounders. Our study cohort was skewed in terms of an overwhelming preponderance of males in patients with esophageal dysplastic/malignant lesions compared with controls. When females were excluded, antibody detection to any high-risk mucosal HPV was significantly greater in cases than controls. Although the sample size was modest, it was somewhat compensated for controls almost three times greater in number. As the serological analysis was site specific, there was little chance that HPV infections elsewhere other than the esophagus may have influenced the prevalence rates. The prospective study design specifically excluded patients with known HPV-associated diseases, post-HPV vaccination, or with a current or previous history of an HPV-associated lesion; immunosuppressed patients; and known HIV positivity. By doing this, we minimized confounding and thus reduced the chances of a spurious association of viral antibodies and BD/EAC. Nevertheless, given that the individuals were not swabbed for oral or genital infections, we cannot definitively exclude the possibility that HPV infections elsewhere could have influenced the result. A limitation of this investigation is a potential viral exposure misclassification, given that not all subjects exposed to HPV seroconvert and that antibody levels can wane over time.[53] We did not have information pertaining to a sexual history of the participants in this study. Nevertheless, in a hospital case–control investigation utilizing a questionnaire-based survey to explore the sexual behavior of 36 BD/EAC patients and 55 controls with known HPV DNA status and markers of transcriptional activity of the esophageal epithelium, we found that HPV exposure conferred a significantly higher risk for BD/EAC as compared with hospital/reflux/BM controls (OR = 6.8, 95% CI: 2.1–23.1, $P = 0.002$ (adjusted)). On the univariate analysis, $\geq$6 lifetime oral sex partners were significantly associated with dysplastic BE and EAC (OR = 4.0; 95% CI: 1.2–13.7, $P = 0.046$). After adjusting for confounders, males with $\geq$2 lifetime sexual partners were at significant risk for BD/EAC.[54] These epidemiological data strengthen the orosexual transmission route postulated previously.[55]

Although antibodies against HPV were generally uncommon in BD/intramucosal EAC, immune responses against two early proteins of HPV16 (E7) and HPV18 (E1) were significantly more frequent in viral-positive dysplastic and early neoplastic lesions of the esophagus. A larger study involving patients with invasive EAC is warranted, though it can be difficult to recruit patients with advanced disease into clinical trials.

Ethics approval and consent to participate

The Human Research Ethics Committee, Tasmania (H9304), and the South Western Sydney Local Health Network (HREC/11/LPOOL/42, LNR/12/LPOOL/221, and LNR/12/LPOOL/131) approved this study. Written informed consent was obtained from each patient prior to participation in this study. This investigation was performed in accordance with the Declaration of Helsinki.

Availability of data and materials

Data will be made available to interested researchers upon request to the South Western Sydney Local Health Network Ethics Committee.

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Author contributions

S.R. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. S.R. conceptualized the study. S.R. and K.H. collected the data. S.R., W.X., K.H., P.S., and B.W formally analyzed the data. S.R. contributed to the funding. S.R., K.H., D.P., N.A., A.R., and B.W. helped with investigation. S.R., W.X., K.H., and B.W. helped with methodology. S.R. handled the project. S.R. collected the resources. S.R. was also involved with supervision. K.H. helped with validation. S.R., K.H., P.S., D.P., N.A., A.R., and B.W. wrote the original draft. S.R., W.X., K.H., P.S., D.P., N.A., A.R., and B.W. wrote, reviewed, and edited the manuscript. The final submitted manuscript was reviewed and approved by all authors.

Competing interests

P.S. has been a consultant for Boston Scientific and currently is one for Olympus, and receives grants from Erbe, US Endoscopy, Ironwood Pharmaceuticals, and Medtronics.

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