

# High-Risk Human Papillomavirus in Esophageal Squamous Cell Carcinoma

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## Abstract

**Background:** Although most cases of esophageal squamous cell carcinoma (ESCC) in western populations have been attributed to high levels of exposure to tobacco and alcohol, infectious agents have been postulated as possible causes, particularly human papillomavirus (HPV).

**Methods:** To explore this issue, we analyzed HPV DNA prevalence and HPV types together with lifestyle factors, in relation to tumor stage and survival in a low-incidence population. Archived tumor samples from a nationwide cohort of 222 ESCC patients were tested for the presence of HPV DNA by PCR; positive samples were sequenced to determine HPV type, and p16<sup>INK4a</sup> status was assessed by immunohistochemistry.

**Results:** Of 222 ESCC patients, 8 tested HPV positive (prevalence, 3.6%; 95% confidence interval, 1.1-6.1%), of which 6 were HPV-16 positive and 2 were HPV-35 positive. Four of the eight HPV-positive tumors over-expressed p16<sup>INK4a</sup>. None of 55 normal esophageal tissue samples from healthy participants had any detectable HPV. Although the numbers were low, it seemed that patients with HPV-positive ESCC tumors were younger than those with HPV-negative tumors (mean age, 60.8 versus 65.3 years,  $P = 0.18$ ) and had higher body mass index (BMI) throughout life (mean current BMI of 25.1 for HPV positive, 22.2 for HPV negative,  $P = 0.08$ ; mean BMI at 20 years of 25.8 for HPV positive, 22.1 for HPV negative,  $P = 0.003$ ). We found no difference between patients with HPV-positive and HPV-negative tumors with respect to other lifestyle factors.

**Conclusions:** These findings suggest a very low prevalence of HPV DNA in human ESCC.

**Impact:** HPV is very unlikely to be a common cause of ESCC in Australia. *Cancer Epidemiol Biomarkers Prev*; 19(8); 2080-7. ©2010 AACR.

## Introduction

Cancer of the esophagus is currently the eighth most common human cancer and the sixth most common cause of cancer-related death (1). In 2002, 462,000 new cases of esophageal cancer were diagnosed worldwide. Globally, squamous cell carcinoma (SCC) is the most common subtype of esophageal cancer, notwithstanding recent increases in the incidence of the adenocarcinoma subtype in western populations (2).

Previous studies have suggested that some chemicals, nutritional deficiencies (3), and excessive use of tobacco and alcohol (2), along with physical factors such as the ingestion of coarse or hot food, may be associated with the development of esophageal SCC (ESCC; refs. 2, 4-7). Infectious agents have also been implicated, as either direct carcinogens or promoters. In particular, human papillomavirus (HPV) has been postulated as a possible cause of ESCC (8). Bovine papillomavirus has been established as a cause of esophageal cancers in cattle (9, 10). HPV DNA has been detected in 30% of human head

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and neck cancers (11-14), and because the esophagus can be infected with these viruses in the same way as the oral cavity, tonsils, and pharynx, it is speculated that HPV infection might explain at least some esophageal cancers in humans. However, evidence for such an association is limited. Previous studies have shown large variations in the prevalence of HPV among patients with ESCC depending on geographic area, ethnic group, and the method used for viral detection (8, 15-20). Even PCR-based studies using similar methodology report varying HPV DNA prevalence in human ESCC tissues (8). Of the HPV types reported in human ESCC specimens, HPV-16 has been the most commonly identified, followed by other high-risk HPV types, including HPV-18, HPV-31, and HPV-35 (16, 21). Serologic analyses of HPV in human esophageal cancer patients have been inconclusive. Several explanations are possible; for example, serologic studies do not distinguish co-incident HPV infection at other body sites from infection in the esophageal squamous epithelium. Moreover, there has been a lack of consistency in the serologic methods used to investigate the association, which might account for the divergent findings of studies (22-25). To date, no firm evidence for a role for HPV in esophageal carcinogenesis has been established.

We analyzed HPV DNA prevalence and HPV types together with lifestyle factors, survival, and tumor stage in an ESCC patient cohort from a geographical low-incidence area, and herewith report the findings.

## Materials and Methods

### Study population and samples

For this study we used data and samples from an Australian nationwide case-control study of esophageal cancer that has been described previously (26).

Briefly, eligible case patients were people ages 18 to 79 years with a histologically confirmed primary invasive cancer of the esophagus or esophagogastric junction diagnosed between July 1, 2002 (July 1, 2001 in Queensland) and June 30, 2005 in the mainland states of Australia. Patients were recruited either through major treatment centers or state-based cancer registries. A total of 315 patients with squamous cell carcinoma of the esophagus gave consent for their tumor tissues to be used in the study. Attempts were made to retrieve paraffin blocks from pathology laboratories at which the 315 ESCC patients were diagnosed; archival sections of formalin-fixed paraffin-embedded tumor tissue were available and released for 237 patients. Of these, pathology review established that samples from 222 patients had sufficient SCC tumor tissue for HPV DNA analysis and p16 immunohistochemistry.

Case participants completed a health and lifestyle questionnaire, which asked about their education, income, occupation, general health, height, and current weight, as well as weight one year ago, at age 20 years, and maximum ever weight. We also elicited a detailed

smoking and alcohol history. Body mass index (BMI) was calculated by dividing the weight in kilograms by the square of height in meters. We derived the lifetime smoking dose in pack-years by summing the smoking dose over each decade of life, calculated by multiplying the decade-specific smoking intensity during that decade (cigarettes/day) by the number of days of smoked per week and the smoking duration within that decade. Details of tumor histology were abstracted from the pathology report accompanying each tumor specimen. Date and cause of death of each case patient in the cohort were obtained through record linkage to the National Death Index (January 15, 2008); complete data were available for 220 patients.

Biopsy samples of normal esophageal squamous epithelium were available from 55 control patients who participated in a parallel study and answered the same questionnaire as the cases. Each of these control patients had undergone upper gastrointestinal endoscopy to investigate dyspepsia or related symptoms; the esophageal tissue samples thus obtained were reported as histologically normal.

This project was approved by the human research ethics committee of the Queensland Institute of Medical Research and participating hospitals, and written informed consent was obtained from all participants.

### PCR analysis

At least one formalin-fixed paraffin-embedded tissue block was available for each of 200 ESCC patients, and a new blade was used to section each block. More precisely, one block per patient was analyzed for 163 patients, two blocks each for 24 patients, three blocks each for 4 patients, four blocks each for 4 patients, five blocks each for 3 patients, and eight blocks each for 2 patients. To minimize the risk of contamination we used different pipettes and rooms for DNA extraction, preparing the PCR solution, adding DNA samples to PCR solution, and electrophoresis analysis. DNA was extracted from three sections (7  $\mu$ m each) per block using the Quick-Extract FFPE DNA Extraction Kit (Epicentre Biotechnologies). For another 22 ESCC patients, only sections on slides were available. For these patients, DNA was extracted from one slide per patient with the same kit. Extraction of DNA with the QuickExtract FFPE DNA Extraction Kit was done by adding 100  $\mu$ L of Quick-Extract FFPE DNA Extraction Solution to the paraffin-embedded tissue sections in an Eppendorf tube; samples were incubated at 56°C for 1 hour and then at 98°C for 2 minutes. The DNA extracted samples were analyzed by PCR for the presence of HPV with the general mucosal HPV primer GP5+/GP6+ (27), and  $\beta$ -globin PCR with the primers PCO3 and PCO4 (28) was carried out on all samples to ensure that they contained human DNA and that no PCR inhibiting agents were present. The final volume of PCR solution (25  $\mu$ L) contained 5  $\mu$ L of extracted sample DNA, 0.5  $\mu$ mol/L of the GP5+ and GP6+ primers or PCO3 and PCO4 primers (Sigma-Aldrich), dNTPs at

concentrations of 0.2 mmol/L each (Roche), 1 U of AmpliTaq Gold DNA polymerase, 1× PCR Gold buffer, and 2.0 mmol/L MgCl<sub>2</sub> (Applied Biosystems). Forty cycles of amplification were done on an Eppendorf Mastercycler Gradient PCR machine after an initial step of 10 minutes denaturation at 94°C. Each amplification cycle for the GP+ PCR consisted of 94°C for 1 minute, 40°C for 2 minutes, and 72°C for 1.5 minutes, plus a final elongation step at 72°C for 4 minutes. Each amplification cycle for the β-globin PCR consisted of 94°C for 1.5 minutes, 50°C for 1.5 minutes, and 72°C for 1.5 minutes, plus a final elongation step at 72°C for 4 minutes. In each batch of tests, H<sub>2</sub>O was used as a negative control. HeLa cells (HPV-18-positive cervical cell line) were used as a positive control in both PCR reactions. PCR amplicons were analyzed by electrophoresis (1.5% agarose gel containing ethidium bromide; SeaKem, FMC bioproducts and Sigma) and identified under UV light. Patients were considered HPV positive if at least one sample tested positive by PCR (37 of the 222 patients, or 17%, had more than one sample analyzed).

### HPV type determination

Fifteen microliters of the HPV-positive PCR products were purified with the Agencourt AMPure PCR purification kit (Agencourt Bioscience) in a magnetic 96-ring SPRIplate. The sequencing reaction contained the purified PCR products together with 3.25 μmol/L of primer and BigDye Terminator (Applied Biosystems). The sequencing reaction was done in an Eppendorf Mastercycler Gradient PCR machine. After an initial step of 96°C for 2 minutes, 20 cycles followed, each with 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 2 minutes. The sequence reactions were purified with the Agencourt CleanSEQ dye-terminator removal kit (Agencourt Bioscience) in a magnetic 96-ring SPRIplate, and purified sequence reactions were analyzed with an automated DNA sequencer (ABI model 3100). The DNA sequences obtained were compared with available sequences in GenBank through the BLAST server (29).

### p16<sup>INK4a</sup> immunohistochemistry analysis

HPV-positive tumor samples underwent immunohistochemical analysis for p16<sup>INK4a</sup> overexpression, a widely accepted test for HPV oncogene expression (30). Tissue sections (4 μm) were mounted onto Superfrost Plus slides and stained with a monoclonal p16<sup>INK4a</sup> antibody (556560) from BD PharMingen according to the protocol recommended by the supplier. A breast carcinoma specimen was used as positive and negative (IgG1 isotype) control in the analysis. All slides were reviewed by a pathologist specializing in gastrointestinal pathology.

### Statistical analysis

We calculated the prevalence and 95% confidence intervals of HPV in ESCC tumors using standard formulae (31). We then sought to compare patients with HPV-positive tumors with those with HPV-negative tumors

to identify characteristics associated with positivity. For comparisons, we used the *t*-test for normally distributed continuous variables and the Wilcoxon test for variables with a skewed distribution. For categorical variables we used the  $\chi^2$  test or Fischer's exact test for small samples when the expected number in any cell was <5. We fitted Cox proportional hazards models to assess whether HPV status of the tumors was associated with survival time, and included terms for age, sex, and stage of the tumor. All analyses were conducted in SAS (V9.1) and all significance tests were two-sided tests at  $\alpha = 0.05$ .

## Results

### Patient characteristics

The mean age for the 222 ESCC patients was 65.2 years (SD, 9.2) and 56% were male, whereas the 55 esophageal tissue controls had a mean age of 52.9 years (SD, 15.9) and 47% were male. Table 1 presents the other salient characteristics. When comparing the 222 patients for whom tissue specimens were available with the 93 consenting patients for whom specimens were not available or HPV tested, we found no difference with respect to age ( $P = 0.24$ ), gender ( $P = 0.70$ ), current BMI ( $P = 0.40$ ), BMI last year ( $P = 0.41$ ), BMI maximum ever ( $P = 0.73$ ), BMI at 20 years ( $P = 0.85$ ), education ( $P = 0.17$ ), income ( $P = 0.12$ ), smoking ( $P = 0.88$ ), alcohol consumption ( $P = 0.47$ ), or American Joint Committee on Cancer (AJCC) cancer stage ( $P = 0.18$ ). However, ESCC patients for whom tumor tissues were available for study had significantly longer survival (median, 2 years and 5 months) than those for whom tissues were not available (median, 1 year and 9 months;  $P = 0.02$ ).

### HPV prevalence, HPV types, and p16<sup>INK4a</sup> immunohistochemistry

Eight (3.6%; 95% confidence interval, 1.1-6.1%) of the 222 ESCC patients were determined to be HPV positive by PCR, and all eight samples were found to contain a high-risk HPV type after sequence confirmation. HPV-16 was the most commonly detected type, being present in six of the eight HPV-positive patients. HPV-35 was detected in tumor samples from a further two patients. No mucosal low-risk HPV types or skin HPV types were found. One tissue sample per patient was available for five of the HPV-positive ESCC patients, and three patients had two samples. One patient with two samples and four of the patients with one sample each tested strongly positive in all samples. Two of the patients with two samples tested positive (weakly) in one sample and negative in the other. All HPV-positive samples were from tissue blocks; no samples from slides tested positive. None of the 55 esophageal tissue controls tested positive for HPV DNA by PCR. All samples in this study tested positive for β-globin by PCR, indicating that there was sufficient DNA to carry out PCR analysis and that no PCR inhibiting agents were present in the samples.

**Table 1.** Selected characteristics of the esophageal cancer patients and the healthy controls

| Characteristics                   | ESCC patients* | Esophageal tissue controls* |
|-----------------------------------|----------------|-----------------------------|
| <i>n</i>                          | 222            | 55                          |
| Age in y, mean (SD)               | 65.2 (9.2)     | 52.9 (15.9)                 |
| Sex, <i>n</i> (%)                 |                |                             |
| Male                              | 124 (56)       | 26 (47)                     |
| Female                            | 98 (44)        | 29 (53)                     |
| Australian born, <i>n</i> (%)     |                |                             |
| Yes                               | 161 (75)       | 26 (65)                     |
| No                                | 55 (25)        | 14 (35)                     |
| BMI, mean (SD)                    |                |                             |
| Now                               | 22.3 (4.7)     | 27.1 (8.2)                  |
| 1 y ago                           | 24.9 (5.1)     | 27.6 (8.7)                  |
| Maximum ever                      | 27.0 (5.2)     | 29.8 (8.4)                  |
| At 20 y                           | 22.3 (3.4)     | 22.1 (3.3)                  |
| Smoking, <i>n</i> (%)             |                |                             |
| Current smoker                    | 68 (31)        | 8 (19)                      |
| Ex-smoker                         | 96 (44)        | 15 (36)                     |
| Nonsmoker                         | 53 (25)        | 19 (45)                     |
| Alcohol consumption, <i>n</i> (%) |                |                             |
| None                              | 25 (12)        | 4 (9)                       |
| <1 drink/wk                       | 14 (6)         | 12 (29)                     |
| 1-6 drinks/wk                     | 49 (23)        | 10 (24)                     |
| 7-20 drinks/wk                    | 39 (18)        | 10 (24)                     |
| ≥21 drinks/wk                     | 87 (41)        | 6 (14)                      |
| Education, <i>n</i> (%)           |                |                             |
| School only                       | 131 (60)       | 22 (49)                     |
| Tech/Diploma                      | 67 (31)        | 18 (40)                     |
| University degree                 | 19 (9)         | 5 (11)                      |
| Income, <i>n</i> (%)              |                |                             |
| ≤AUD 30K                          | 109 (60)       | 27 (69)                     |
| AUD 30K-AUD 60K                   | 53 (29)        | 9 (23)                      |
| ≥AUD 60K                          | 21 (11)        | 3 (8)                       |

\*Numbers may not sum to total due to missing data.

Four of the eight HPV-positive ESCC patients stained positive for p16<sup>INK4a</sup> with immunohistochemistry (Table 2). Three of the four ESCC tumors that were negative for p16<sup>INK4a</sup> were weakly HPV positive, whereas the other showed a moderately strong PCR band. The four ESCC samples that were p16<sup>INK4a</sup> positive ranged from weakly to strongly HPV positive.

#### Patient characteristics associated with HPV-positive and HPV-negative ESCC tumors

HPV-positive ESCC patients were, on average, younger than HPV-negative patients (60.8 and 65.3 years, respectively), although this difference was not statistically significant ( $P = 0.18$ ; Table 3). There was evidence that patients with HPV-positive ESCC had higher BMI than those with HPV-negative ESCC. The measure of BMI at 20 years was significantly higher in the HPV-positive than in HPV-negative patients ( $P = 0.003$ ), whereas the

difference in current BMI was of marginal statistical significance ( $P = 0.08$ ). Measures of BMI one year ago and maximum BMI were also higher for the HPV-positive patients, although these differences were not statistically significant. There was no statistically significant difference between the patients with HPV-positive ESCC tumors and the HPV-negative group with respect to gender, country of birth, smoking, alcohol consumption, education, or income (Table 3).

Survival data were available for 220 of the ESCC cases (213 HPV-negative and 7 HPV-positive patients). Two of the HPV-positive patients and 136 of the HPV-negative patients died. On crude analysis, we found longer median survival for patients with HPV-positive (mean survival, 3 years and 7 months) than HPV-negative (mean survival, 2 years and 8 months) ESCC tumors ( $P = 0.17$ ), although this was not statistically significant. When further assessed using proportional hazard models,

which included terms for age, sex, and stage, there was no evidence of survival advantage associated with HPV status (hazard ratio 0.9; 95% confidence interval, 0.3-2.8).

When AJCC tumor stage was analyzed at discrete levels (0, I and IIA, IIB and III, and IV), we found significant differences between HPV-positive and HPV-negative ESCC tumors ( $P = 0.0009$ ), but the data were very sparsely distributed. We recategorized the patients into groups of low (0, I, and IIA) and high (IIB, III, and IV) stages, and found no difference in stage distribution between HPV-positive and HPV-negative tumors ( $P = 0.40$ ).

## Discussion

We observed a very low prevalence of HPV DNA in ESCC tumors in this sample of Australian patients with ESCC. We found no evidence of HPV DNA in any of the 55 samples of normal esophageal squamous epithelium. An extensive review by Syrjänen reported the average prevalence of HPV DNA in ESCCs to be 15% using PCR (8). However, reports of prevalence of HPV in ESCC vary greatly between geographic areas, and between areas of high and low incidence for esophageal carcinoma (8). Our finding of a very low prevalence of HPV DNA in ESCC tumors was similar to observations from a number of other studies conducted among populations with a low incidence of ESCC, including Korea (0% HPV prevalence), Slovenia (0%), Italy (0%), and the United States (2%; refs. 19, 32-34). Slightly higher estimates of HPV prevalence have been reported from other populations with low ESCC incidence, including Shandong (6%), Sweden (16%), and Germany (17%; refs. 15, 17, 21). In populations with higher ESCC incidence rates, reports of HPV prevalence in tumor tissues have ranged from 0% in Kenya and Linxian Province in China (18, 35), to 54%, 65%, and 72% in Egyptian, Gansu (China), and Anyang (China) populations, respectively (15, 36, 37).

**Table 2.** Summary of HPV-positive patients and their HPV types together with p16<sup>INK4a</sup> status

| Patient | HPV positivity | HPV type | p16 <sup>INK4a</sup> expression |
|---------|----------------|----------|---------------------------------|
| 1       | (+)            | HPV-16   | NEG                             |
| 2       | ++             | HPV-16   | NEG                             |
| 3       | ++             | HPV-35   | POS                             |
| 4       | +++            | HPV-16   | POS                             |
| 5       | ++             | HPV-16   | POS                             |
| 6       | (+)            | HPV-16   | NEG                             |
| 7       | ++             | HPV-16   | POS                             |
| 8       | (+)            | HPV-35   | NEG                             |

NOTE: (+), weak; +, moderate; ++, strong; +++, very strong positivity.

Abbreviations: NEG, negative; POS, positive.

The two HPV types identified in our study (HPV-16 and HPV-35) are both high-risk HPV types (potentially cancer causing; ref. 14), with HPV-16 being the most commonly detected HPV type. According to previous studies, HPV-16 has been by far the most prevalent HPV type in esophageal cancer patients globally, usually followed by HPV-18 as the second most prevalent HPV type (16, 17, 21, 37).

Only single HPV infections were identified in our study population. This seems to be a consistent finding in most studies, although double or even multiple HPV infections have been reported in ESCC tumors from some areas of China (37).

Two patients in our study with two samples were weakly positive in one sample and negative in the other, which could reflect low viral load. Viral load in esophageal cancer has previously been shown to vary greatly, with a range from 1 HPV copy/100,000 genomes up to 150 copies/genome (38).

Overexpression of cellular p16<sup>INK4a</sup> has previously been linked with HPV oncogene activity, especially in cervical cancer (30, 39), and has been used as a confirmatory test (40). We found that four of our eight HPV-positive ESCC cases overexpressed p16<sup>INK4a</sup>. Assuming that the co-occurrence of HPV DNA by PCR and p16<sup>INK4a</sup> staining by immunohistochemistry results from HPV infection of esophageal cells, then our data suggest that HPV may be causally associated with no more than 2% of squamous cell cancers of the esophagus.

Of the lifestyle factors analyzed, we found some evidence that patients with HPV-positive ESCC had higher BMI than patients with HPV-negative tumors. Reporting bias cannot explain this finding, because participants and investigators were unaware of the HPV status of the tumors at the time of data collection. Moreover, the finding of higher BMI among HPV-positive patients was consistently observed at all time points, albeit not always at the level of statistical significance. Large prospective studies have consistently observed that people who go on to develop ESCC are typically lean throughout their adult life, and it has been widely assumed that this reflects the high levels of smoking and alcohol intake associated with the majority of ESCC patients in western populations (41-43). Our sample was too small to confidently exclude confounding by these factors, although there was no apparent difference in the prevalence of smoking or alcohol use among patients with HPV-positive versus HPV-negative tumors.

As has been reported for SCC of the head and neck (44), we observed that patients with HPV-positive ESCC were younger than their HPV-negative counterparts, although this finding was not statistically significant. To our knowledge, this is the first time that the possibility of differences in age at onset has been reported for esophageal cancer by HPV status, and warrants confirmation in other datasets.

Although on crude analysis there was some suggestion that patients with HPV-positive tumors had longer

**Table 3.** Characteristics of HPV-positive and HPV-negative ESCC patients

| Characteristics                   | HPV-positive patients* | HPV-negative patients* | P     |
|-----------------------------------|------------------------|------------------------|-------|
| <i>n</i>                          | 8 (3.6)                | 214 (96.4)             |       |
| Age in y, mean (SD)               | 60.8 (10.0)            | 65.3 (8.4)             | 0.18  |
| Sex, <i>n</i> (%)                 |                        |                        |       |
| Male                              | 4 (50)                 | 120 (56)               | 0.73  |
| Female                            | 4 (50)                 | 94 (44)                |       |
| Australian born, <i>n</i> (%)     |                        |                        |       |
| Yes                               | 5 (63)                 | 156 (75)               | 0.42  |
| No                                | 3 (37)                 | 52 (25)                |       |
| BMI, mean (SD)                    |                        |                        |       |
| Now                               | 25.1 (6.1)             | 22.2 (4.6)             | 0.08  |
| 1 y ago                           | 26.4 (5.4)             | 24.8 (5.1)             | 0.39  |
| Maximum ever                      | 29.2 (4.4)             | 26.9 (5.3)             | 0.22  |
| At 20 y                           | 25.8 (4.0)             | 22.1 (3.3)             | 0.003 |
| Smoking, <i>n</i> (%)             |                        |                        |       |
| Current smoker                    | 2 (25)                 | 66 (32)                | 0.54  |
| Ex-smoker                         | 5 (62)                 | 91 (43)                |       |
| Nonsmoker                         | 1 (13)                 | 52 (25)                |       |
| Alcohol consumption, <i>n</i> (%) |                        |                        | 0.88  |
| None                              | 1 (12)                 | 24 (12)                |       |
| <1 drink/wk                       | 1 (13)                 | 13 (6)                 |       |
| 1-6 drinks/wk                     | 2 (25)                 | 47 (23)                |       |
| 7-20 drinks/wk                    | 2 (25)                 | 37 (18)                |       |
| ≥21 drinks/wk                     | 2 (25)                 | 85 (41)                |       |
| Education, <i>n</i> (%)           |                        |                        |       |
| School only                       | 4 (50)                 | 127 (61)               | 0.62  |
| Tech/Diploma                      | 3 (38)                 | 64 (31)                |       |
| University degree                 | 1 (12)                 | 18 (8)                 |       |
| Income, <i>n</i> (%)              |                        |                        |       |
| ≤AUD 30K                          | 4 (57)                 | 105 (60)               | 0.97  |
| AUD 30K-AUD 60K                   | 2 (29)                 | 51 (29)                |       |
| ≥AUD 60K                          | 1 (14)                 | 20 (11)                |       |

\*Numbers may not sum to total due to missing data.

survival than those with HPV-negative tumors, we found no evidence of survival advantage once we adjusted for other confounding factors in proportional hazards models. Two previous smaller Scandinavian studies also did not find any associations between HPV status and survival in esophageal cancer patients (17, 45).

For SCC of the head and neck, HPV-positive tumors have been reported to have better responses to treatment and increased sensitivity to radiotherapy, leading to the suggestion that HPV infection makes the tumors more susceptible to treatment (11, 46, 47). Here we found that HPV-positive tumors were of a significantly higher histologic AJCC stage at discrete levels. However, due to low numbers of HPV-positive tumors the stages were recategorized as high or low stage, and no significant difference with regard to HPV status was identified. Again, the overall low prevalence of HPV-positive tumors in this study precluded robust statistical analysis.

The strengths of the current study include the large sample of cases, the systematic collection of detailed health and lifestyle information prior to the ascertainment of tumor tissues, the control series of normal esophageal tissue samples, and the follow-up of cases through the National Death Index. In addition, although there is no universally accepted "gold standard" primer set for detecting HPV DNA in formalin-fixed, paraffin-embedded tissues, the GP5+/GP6+ primers we used have been shown to be highly sensitive for detecting HPV DNA in exfoliated cervical cells (48), and have been used previously for detecting HPV DNA in formalin-fixed tissues (49). Taken together, these features give confidence that the prevalence estimate and reported associations are unlikely to be biased by the selection of participants or the collection of data. However, although the sample was relatively large, our study was underpowered to fully explore the associations with possible explanatory factors.

Pooling these data with similar studies would overcome this limitation and permit more detailed analyses.

In conclusion, our findings suggest that HPV DNA is rarely present in the tumors of Australian patients with ESCC. There is weak evidence that these patients are younger and have a higher BMI. Given the low HPV DNA prevalence in this study, larger studies or pooled analyses will be required for definitive evidence regarding the role of HPV in squamous cell carcinomas of the human esophagus.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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