A Prospective, Seroepidemiological Study of the Role of Human Papillomavirus in Esophageal Cancer in Norway

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ABSTRACT

Infection with the human papillomavirus (HPV), notably HPV type 16, has been associated with esophageal cancer in seroepidemiological studies. To evaluate the consistency of the association, we performed a nested case-control study of HPV seropositivity and risk of esophageal cancer within a prospectively followed cohort of 300,000 Norwegian men and women who had donated blood samples to a serum bank. The data file of the serum bank was linked with the nationwide Cancer Registry of Norway to identify esophageal cancers diagnosed after donation of the serum sample. Fifty-seven cases and 171 matched controls were analyzed for antibodies to specific microorganisms, and odds ratios for developing esophageal cancer were calculated. There was an increased risk of developing esophageal cancer among HPV 16-seropositive subjects (odds ratio = 6.6; 95% confidence interval, 1.1—71) but not among Chlamydia trachomatis-seropositive subjects. Adjustment for the presence of cotinine, a marker of smoking habits, did not affect the estimates substantially. The seroepidemiological association between HPV 16 and esophageal cancer seems to be consistent in different countries.

INTRODUCTION

Esophageal cancer is the ninth most common neoplasm worldwide, but it is the sixth most common cause of cancer death, responsible for 287,000 deaths annually (1, 2). The incidence of esophageal cancer shows a wide geographic variation (3). Generally, the incidence rates are much higher in men than in women. In the Nordic countries, from 1983 to 1987, the age-adjusted (world standard population) incidence rates were 3.3 per 100,000 person-years in men and 1.2 per 100,000 person-years in women. The median ages of the patients were 70 years for men and 75 years for women (4). In Norway, the incidence rates of esophageal cancer have been stable at a lower level the last 40 years (287,000 deaths annually). The median ages of the patients were 70 years for men and 75 years for women (4). In Norway, the incidence rates of esophageal cancer have been stable at a lower level the last 40 years (287,000 deaths annually).

Epidemiological and experimental studies have suggested that smoking, excessive alcohol consumption, low socioeconomic status, specific chemical agents, and nutritional deficiencies are major risk factors for esophageal cancer, but these risk factors have not explained the geographic variation of the disease (5). A HPV3 involvement in the etiology of esophageal cancer was first suggested by Syrjänen in 1982 (6). Subsequently, several studies have reported the presence of HPV DNA, most often type 16, in esophageal tumors (7–9). However, HPV DNA positivity has varied considerably in different studies (10).

HPV serology, using capsids of HPV 16, 18, and 33, has been extensively validated as a type-restricted marker of past or present HPV infection (11). Use of HPV serology has previously provided both prospective and cross-sectional epidemiological evidence of a link between HPV 16 and esophageal cancer (12, 13).

In Norway, the Janus serum bank was established in the early 1970s, and a population-based cancer registry has been in operation since the early 1950s. Here, we used these data sources for a prospective, seroepidemiological evaluation of the risk of developing esophageal cancer following infection with HPVs (types 16, 18, and 33). Seropositivity for Chlamydia trachomatis was determined to control for environmental exposures secondarily associated with a lifestyle that includes sexual risk-taking behavior.

MATERIALS AND METHODS

The Janus Project. The Janus serum bank (14) was initiated in 1973, and it contains approximately 500,000 serum samples, stored at −25°C, from about 300,000 donors. The specimens have been collected from individuals who participated in county health examinations, mostly for cardiovascular diseases, and from blood donors. The participants in the health examinations were recruited from several counties in various parts of Norway. The blood donors were from the Red Cross Blood Donor Center in Oslo. In 1997, these donors ranged in age from 27 to 89 years, whereas most of the other donors were in the age range of 55–75 years.

The Cancer Registry of Norway. Since 1953, the Cancer Registry of Norway has received information on all cancer patients in the population. The reporting system is based on pathology and cytology reports, clinical records, and death certificates and provides information about site, histological type, and stage of disease at the time of diagnosis. Also, the 11-digit individual identification number allocated to every resident of Norway is reported. The registration of solid cancers is regarded as practically complete (15). Registration is based on a modified version of International Classification of Diseases, 7th revision.

Identification of Cases and Controls. On the basis of the personal identification number, the data files of the serum bank and the cancer registry were linked to identify cases with esophageal cancer. If there were several serum samples available per case, the first sample was chosen. Three controls were selected from the cohort for each case. The controls were individually matched for sex, age at serum sampling (±2 years), storage time (±2 months), and county of residence. If three controls could not be found, the matching criteria were expanded for sex, age at serum sampling (±2 years), storage time (±2 months), and county of residence. If three controls could not be found, the matching criteria were expanded for sex, age at serum sampling (±2 years), storage time (±2 months), and county of residence. Initial, 58 cases and 174 controls were identified. The serum sample was not available for one case. Consequently, a total of 57 cases (52 men and 5 women) and 171 controls were left for analysis. The matching criteria were expanded for three controls. Altogether, 41 cases were squamous cell carcinomas, 9 were adenocarcinomas, 4 were other histological types, and 3 were not histologically verified.

The median age at diagnosis was 59 years (range, 43–73 years). Median
time between withdrawal of serum and diagnosis was 14 years (range, 0.9–21 years).

**Laboratory Methods.** Seropositivity to HPV capsids was determined by a previously established and validated ELISA using baculovirus-expressed capsids, comprising both the L1 and L2 proteins, with disrupted capsids of bovine papillomavirus as control (16). Seropositivity was assessed using two cutoff levels, which both have been used in previous studies (16–18). Both primary and alternative cutoff levels, as well as their status as primary or alternative, were assigned in advance of the analyses. To avoid the possibility of finding spurious associations in analyses of multiple different cutoffs, the data were analyzed for the two preassigned cutoff levels only. A cutoff of 0.100 absorbance units distinguishes HPV 16-infected and sexually inexperienced women (18). For HPV 16, analysis was also performed using a cutoff level of 0.239 (primary cutoff level), which, relative to internal standards, was the same as the one giving optimal discrimination of cases and controls in a previous study of cervical cancer (19). Comparability of analyses in different runs and with analyses in previous studies was ensured by analysis of a panel of internal standard serum pools (with ELISA absorbances in the linear part of the absorbance curve) on each plate. Compared with the analyses of Dilner et al. (12), the analyses of the internal standards in this study had absorbances that were 33% higher than in the previous study. Hence, the primary cutoff level was raised by 33% (from 0.180 to 0.239). Such disease-optimal cutoffs have not been previously established for HPV 18 and HPV 33, and therefore, an arbitrary alternative cutoff level of 0.200 was also analyzed. The specificity of the HPV-serological assays for sexually transmitted HPV infections was high because no antibodies could be found in a panel of sera obtained from virginal women (18). The high correlation with the lifetime (rather than with recent) number of sexual partners implies that serology is a marker of lifetime cumulative exposure. Estimates of sensitivity for detection of HPV 16 infection have, in several previous validation studies, been found to be about 50% (18, 20–22). The HPV type-specificity of the assay has been established for HPV 16 (20, 22), but in the case of the HPV 18 and 33 assays, cross-reactivities with other sexually transmitted HPV types cannot be excluded.

IgG antibodies to *C. trachomatis* were determined by the microimmunoassay method using the following *C. trachomatis* serovars: B, E, D/C, H, I, J, G, F, and K (23). A combination of the results obtained for the different serovars with a preassigned cutoff level of ≥1:16 was used to distinguish seropositivity and seronegativity. *Chlamydia pneumoniae* serovar K6P (cutoff level of ≥1:32) served as a control antigen.

Plasma cotinine was measured in blinded case-control quadruplicate sets by an ELISA microplate method (STC Diagnostics), originally developed as a qualitative test but optimized in the laboratories of the Clinical Trial Service Unit and Epidemiological Studies Unit as a quantitative test in collaboration with the distributor. Anticotinine antibody is coated on 96-well microtiter plates, and the enzyme conjugate is cotinine-labeled with horseradish peroxidase, which is diluted in a protein matrix. Careful testing of this method revealed excellent reliability with correlations to established gas chromatography and RIA methods of 0.95 and 0.94 across cotinine ranges from nonsmokers to heavy smokers, respectively, and of 0.96 and 0.58 across cotinine ranges from nonsmokers to very light smokers, respectively (24, 25). The serum cotinine level was used to separate the subjects into the following four categories: nonsmokers (0–4.99 ng/ml), those exposed to passive smoke (5.0–24.99 ng/ml), lighter smokers (25.0–99.99 ng/ml), and heavy smokers (≥100 ng/ml).

All laboratory analyses were performed with masked samples.

**Statistical Analyses.** ORs and their 95% CIs were derived from conditional exact logistic regression models (26). The data were analyzed using the program LogXact (version 1.1; Cytel Software Corporation, Cambridge, MA).

**RESULTS**

Overall, 21% of the esophageal cancer cases and 11% of the controls were seropositive for HPV 16, 18, or 33. There was an increased risk for developing esophageal cancer among HPV 16-seropositive subjects (OR = 6.6; 95% CI = 1.1–71) at the preassigned cutoff level of 0.239 (Table 1). The association with HPV 18 was not significant. There was an increased risk of developing esophageal cancer among HPV 33-seropositive subjects at the alternative cutoff level of 0.200. The risk of developing esophageal cancer increased with increasing serum cotinine level, but not significantly (P for trend = 0.07). Adjustment for serum cotinine (cutoff level, ≥25 ng/ml) did not affect the estimates substantially. Adjustment with a four-level cotinine variable yielded very similar results. No association with *C. trachomatis* or *C. pneumoniae* was found. Adjustment for both serum cotinine (cutoff level, ≥25 ng/ml) and *C. trachomatis* also did not affect the estimates substantially.

A higher OR among HPV 16-seropositive subjects was observed when the analysis was restricted to squamous cell carcinomas (OR = 10; 95% CI = 1.0–510; Table 2). No significant association with HPV 18 or 33 was seen. The OR for serum cotinine (cutoff level, ≥25 ng/ml) was 4.0 (95% CI = 1.3–17). Adjustment for serum cotinine did not affect the estimates substantially.

The risk of developing esophageal cancer was higher among the HPV-seropositive subjects (seropositive to one or several HPV types) who were younger than 60 years at the time of diagnosis (OR = 3.7; 95% CI = 1.1–13), whereas no association was seen for older subjects (OR = 1.0; 95% CI = 0.2–4.0).

No substantial difference in the ORs of esophageal cancer for HPV seropositivity was noted with a lag time (time between serum sam-

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**Table 1 ORs and 95% CIs of esophageal cancer according to the presence of IgG antibodies to different microorganisms, with and without adjustment for cotinine (smoking)**

<table>
<thead>
<tr>
<th>Cases</th>
<th>Controls</th>
<th>% positive</th>
<th>Without adjustment</th>
<th>With adjustment (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 57)</td>
<td>(n = 171)</td>
<td>OR 95% CI</td>
<td>OR 95% CI</td>
<td>OR 95% CI</td>
</tr>
<tr>
<td><strong>HPV 16</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutoff level, 0.239</td>
<td>9</td>
<td>2</td>
<td>6.6</td>
<td>1.1–71</td>
</tr>
<tr>
<td>Cutoff level, 0.100</td>
<td>12</td>
<td>5</td>
<td>2.8</td>
<td>0.8–9.4</td>
</tr>
<tr>
<td><strong>HPV 18</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutoff level, 0.100</td>
<td>12</td>
<td>6</td>
<td>2.2</td>
<td>0.7–6.6</td>
</tr>
<tr>
<td>Cutoff level, 0.200</td>
<td>11</td>
<td>5</td>
<td>2.2</td>
<td>0.6–7.4</td>
</tr>
<tr>
<td><strong>HPV 33</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutoff level, 0.100</td>
<td>12</td>
<td>6</td>
<td>2.3</td>
<td>0.7–7.3</td>
</tr>
<tr>
<td>Cutoff level, 0.200</td>
<td>12</td>
<td>3</td>
<td>4.8</td>
<td>1.2–23</td>
</tr>
<tr>
<td><strong>HPV 16/18/33</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. trachomatis</em></td>
<td>21</td>
<td>11</td>
<td>2.1</td>
<td>0.9–5.0</td>
</tr>
<tr>
<td><em>C. pneumoniae</em></td>
<td>12</td>
<td>19</td>
<td>0.6</td>
<td>0.2–1.5</td>
</tr>
<tr>
<td><strong>Cotinine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposed to passive smoke (5.0–24.99 ng/ml)</td>
<td>14</td>
<td>20</td>
<td>1.4</td>
<td>0.3–10</td>
</tr>
<tr>
<td>Lighter smokers (25.0–99.99 ng/ml)</td>
<td>4</td>
<td>4</td>
<td>1.9</td>
<td>0.1–25</td>
</tr>
<tr>
<td>Heavy smokers (≥100 ng/ml)</td>
<td>77</td>
<td>65</td>
<td>2.6</td>
<td>0.7–15</td>
</tr>
</tbody>
</table>

\( ^a\) Adjusted for a two-level cotinine variable (cutoff level, ≥25 ng/ml).

\( ^b\) HPV 16: cutoff level, 0.239 HPV 18: cutoff level, 0.100; HPV 13: cutoff level, 0.100.
pluing and diagnosis) of less than (OR = 2.7; 95% CI = 0.6–12) or more than (OR = 1.8; 95% CI = 0.5–5.7) 10 years.

DISCUSSION

In the present prospective, seroepidemiological study, the risk of developing esophageal cancer among subjects who were seropositive to HPV types 16, 18, and 33 was evaluated. An increased risk of esophageal cancer was found for HPV 16-seropositive subjects. To allow for the assessment of possible confounding by known or unknown risk factors secondarily associated with a lifestyle that carries with it an increased risk of HPV infection, the risk associated with C. trachomatis seropositivity was evaluated as a marker of sexual behavior. However, no association was found.

The role of HPVs in the development of anogenital cancers, in particular cervical cancer, has been firmly established. Consistent associations have been reported for other tumors of the genital tract well (10). However, the prevalence of HPVs in these tumors appears to be lower than in cervical cancer. HPVs, especially HPV 16, have also been associated with nongenital cancers, such as cancers of the upper digestive and respiratory tract. In particular, HPV infection has recently received considerable attention as a possible risk factor for esophageal cancer. In a Norwegian study on patients with previous carcinoma in situ of the uterine cervix, an increased risk of developing secondary cancers at specific sites, such as vulvar, vaginal, anal, and esophageal cancer, was found, suggesting the existence of shared risk factors (27).

HPV DNA has been detected in invasive as well as in normal or hyperplastic epithelium adjacent to squamous cell carcinomas in esophagus. HPV 16 and 18 are the most common types found in esophageal squamous cell carcinomas. HPV DNA positivity has, however, varied considerably in different series (10). HPV prevalences found in studies using in situ hybridization range between 0 and 43%. More recent studies using PCR detection are generally reporting 0–10% HPV positivity.

The discrepant findings of the DNA-based studies might have resulted from different etiologies of the disease or from differences in the sensitivity or specificity of the methods used. The complexity of related virus types and PCR contamination are further problems. Detection of HPV DNA is also subject to sampling errors, and lack of comparable biopsy material from cases and controls has limited the ability to evaluate the risk associated with HPV. These methods detect current infection only; prior exposure is not necessarily reflected. By applying HPV serology, a marker of both past or present HPV infection, it has been possible to investigate possible associations of HPV infection with human cancers using standardized sampling and detection methods in reliable study designs.

The possibility that perhaps HPV 16 has a “hit and run” mechanism for inducing esophageal cancer should be considered. In HPV 16-associated cervical cancer, there is a requirement for continued expression of the transforming proteins (E6 and E7) of the virus for continued growth of the tumor (28). A very different situation has been found for esophageal cancer in cattle, which develops from bovine papillomavirus type 4-associated premalignant lesions but which has lost the viral DNA (29). Consequently, papillomavirus exposure appears to be able to increase esophageal cancer risk without the maintenance of the viral genome in the tumor, at least in this animal system.

The serological association between HPV 16 and esophageal cancer has been evaluated in a Finnish prospective, nested case-control study and in a hospital-based case-control study from Shandong Province, China (12, 13). In the low-risk Finnish population, the OR of esophageal cancer was 14.6 for HPV 16, whereas the OR was 4.5 in the high-risk Chinese population. In the present study of a low-risk Norwegian population, the OR of esophageal cancer among HPV 16 seropositive subjects was 6.6. However, the OR increased to a value of 10 when the analysis was restricted to squamous cell carcinomas. Only 9% of the cases and 2% of the controls were seropositive for HPV 16 in this study, compared with 21 and 3% in the Finnish study and 24 and 7% in the Chinese study, respectively.

In the Chinese study, the OR for esophageal cancer among the HPV 16-seropositive subjects increased progressively with higher cutoff levels for seropositivity (13). Persistent infections repeatedly detected on follow-up induce higher levels of HPV antibodies than do transient infections only detected once (30). In the present study, the OR seemed to increase with increasing cutoff levels for HPV 16 and 33, implying an increased risk with a persistent infection (past or present). The preassigned cutoff level of 0.239 is (relative to internal standards) identical to the cutoff level of 0.180 used in the Finnish study (12).

Among men in Western populations, excessive alcohol and tobacco consumption have dominant roles in the etiology of esophageal cancer (5). Drinking and smoking habits and low socioeconomic status have been associated with sexual behavior and thus also with HPV 16 infection at least in some populations (31). In the present study, only heavy smoking (cutoff level, ≥100 ng/ml) was associated with risk, and this association was only significant for squamous cell carcinomas. Further, there was no association with C. trachomatis, a surrogate marker of sexual risk-taking behavior.

The active smokers among the controls (cutoff level, ≥25 ng/ml)

Table 2. ORs and 95% CIs of esophageal squamous cell carcinoma according to the presence of IgG antibodies to different microorganisms, with and without adjustment for cotinine (smoking).

<table>
<thead>
<tr>
<th>% positive</th>
<th>Without adjustment</th>
<th>With adjustment*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases (n = 41)</td>
<td>Controls (n = 123)</td>
</tr>
<tr>
<td>HPV 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutoff level, 0.239</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Cutoff level, 0.100</td>
<td>2.3</td>
<td>0.5–9.1</td>
</tr>
<tr>
<td>HPV 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutoff level, 0.100</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>Cutoff level, 0.200</td>
<td>1.9</td>
<td>0.5–6.5</td>
</tr>
<tr>
<td>HPV 33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutoff level, 0.100</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>Cutoff level, 0.200</td>
<td>1.2</td>
<td>3</td>
</tr>
<tr>
<td>HPV 16/18/33#</td>
<td>24</td>
<td>12</td>
</tr>
<tr>
<td>C. trachomatis</td>
<td>0.7</td>
<td>0.2–1.8</td>
</tr>
<tr>
<td>C. pneumoniae</td>
<td>1.4</td>
<td>0.6–3.3</td>
</tr>
<tr>
<td>Cotinine, ≥25 ng/ml</td>
<td>90</td>
<td>70</td>
</tr>
</tbody>
</table>

* Adjusted for a two-level cotinine variable (cutoff level, ≥25 ng/ml).
# HPV 16: cutoff level, 0.239; HPV 18: cutoff level, 0.100; HPV 13: cutoff level, 0.100.
seemed to be *C. trachomatis* seropositive more often than the others (OR = 2.3; 95% CI = 0.8—7.2), but the active smokers did not seem to be more often positive for HPV than the others (OR = 0.6; 95% CI = 0.2—1.8). Accordingly, adjustment of the HPV 16-associated risk for smoking and for *C. trachomatis* did not affect the estimates substantially, even when smoking was adjusted for as a four-level exposure variable (data not shown). Also, in the previous Finnish seroepidemiological study, adjustment for smoking habits did not affect the HPV 16-related risk substantially (12).

In summary, this study provides prospective, seroepidemiological evidence indicating that infection with HPV 16 confers an increased risk for subsequent development of esophageal cancer. The risk was not affected by adjustment for biomarkers of sexual behavior or for smoking exposure at different levels.

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*Cancer Res* 1997;57:3989-3992.

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